# PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

| (51) International Patent Classification 6 :<br><br>C12N 15/74, C07K 14/315, C12N 9/14, A23C 19/032 | A1 | (11) International Publication Number: WO 95/31562 |
|---|---|---|
| | | (43) International Publication Date: 23 November 1995 (23.11.95) |

<table>
<tr><td>

(21) International Application Number: PCT/NL95/00171

(22) International Filing Date: 12 May 1995 (12.05.95)

(30) Priority Data:<br>
94201354.1     12 May 1994 (12.05.94)    EP<br>
(34) Countries for which the regional or<br>
    international application was filed:    NL et al.

</td><td>

(81) Designated States: AU, JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
*With international search report.*
*Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.*

</td></tr>
</table>

(71) Applicant (for all designated States except US): QUEST INTERNATIONAL B.V. [NL/NL]; Huizerstraatweg 28, NL-1411 GP Naarden (NL).

(72) Inventors; and
(75) Inventors/Applicants (for US only): NAUTA, Arjen [NL/NL]; Helpermolenstraat 37, NL-9721 BT Groningen (NL). VENEMA, Gerard [NL/NL]; Kerklaan 26, NL-9751 NN Haren (NL). KOK, Jan [NL/NL]; Van Hamelstraat 34, NL-9714 HL Groningen (NL). LEDEBOER, Aat, M. [NL/NL]; Montignyplein 8, NL-3955 NL Rotterdam (NL).

(74) Agent: DE BRUIJN, Leendert, C.; Nederlandsch Octrooibureau, Scheveningseweg 82, P.O. Box 29720, NL-2502 LS The Hague (NL).

(54) Title: PROCESS FOR INHIBITING THE GROWTH OF A CULTURE OF LACTIC ACID BACTERIA, AND OPTIONALLY LYSING THE BACTERIAL CELLS, AND USES OF THE RESULTING LYSED CULTURE

(57) Abstract

The invention provides a process for inhibiting the growth of a culture of lactic acid bacteria, or a product containing such culture e.g. a cheese product, in which in the cells of the lactic acid bacteria a holin obtainable from bacteriophages of Gram-positive bacteria, esp. from bacteriophages of lactic acid bacteria is produced in situ, the gene encoding said holin being under control of a first regulatable promoter, said holin being capable of exerting a bacteriostatic effect on the cells in which it is produced by means of a system, whereby the cell membrane is perforated, while preferably the natural production of autolysin is not impaired. It is preferable that additionally a lysin obtainable from lactic acid bacteria or their bacteriophages is produced in situ in the cells of the lactic acid bacteria, the gene encoding said lysin being under control of a second regulatable promoter, whereby the produced lysin effects lysis of the cells of the lactic acid bacteria. The second regulatable promoter can be the same as the first regulatable promoter and the genes encoding the holin and the lysin, respectively can be placed under the same regulatable promoter in one operon. Preferably the promoters are regulatable by the food-grade ingredients or parameters. Other uses of the invention include preparing a mixture of peptides which are modified by peptidases freed after the lysis, using the lysed culture as a bactericidal agent against spoiling bacteria or pathogenic bacteria for improving the shelf life of a product containing the lysed culture.

Process for inhibiting the growth of a culture of lactic acid bacteria,
and optionally lysing the bacterial cells, and uses of the resulting
lysed culture

5

## Background of the invention and prior art

The invention relates to a process for inhibiting the growth of
a culture of lactic acid bacteria, and optionally lysing the cells of
10    said bacteria.

In this specification the following abbreviations of names of
micro-organisms are used:

E. = Escherichia, e.g. E. coli,

L. = Lactococcus, e.g. L. lactis,

15    M. = Micrococcus, e.g. M. lysodeikticus,

S. = Streptococcus, e.g. S. faecalis and S. pneumonia.

In one aspect the invention relates to a process for the lysis
of a culture of lactic acid bacteria, or a product containing such
culture, by means of a lysin e.g. in producing a fermented food product,
20    e.g. in cheese-making. Such a process is known from WO 90/00599
(AGRICULTURAL & FOOD RESEARCH COUNCIL (AFRC), M.J. Gasson, published 25
January 1990, ref. 1). According to that patent specification the lysin
from a Lactococcus (preferably prolate-headed) bacteriophage was used to
lyse bacterial starter cultures during cheese-making. Exemplified was the
25    lysin of the bacteriophage φvML3 of Lactococcus lactis ML3. In
particular, the lysin can be added to a cheese product or a cheese
precursor mixture, e.g. after whey removal, milling and salting. However,
this solution has the disadvantage that thorough mixing of the contents
of the lysed cells with the cheese product is not easily obtained.
30    Another disadvantage is that the lysin was produced by Escherichia coli
cells, which are not food-grade. It is explicitly stated if the cell wall
of the host cell is not itself degraded by the lysin then the lysin
secreting transformed host may be useful in suppressing  populations of
bacteria which are susceptible to lysis by the lysin. Nothing is
35    mentioned regarding addition of a transformed host cell in improving
chees flavor, certainly not a transformed lactic acid bacterium.

As an alternative it is suggested in that patent specification
"to encapsulate the lysin so that the timing of its
addition is not important. The encapsulating agent
40              dissolves after the cheese-making process is complete

thus not affecting the starter bacteria before their
role in acidification was complete."

This suggested alternative has the disadvantages, that (a) an
encapsulating material has to be used, and (b) said material must not
5    dissolve before the end of the cheese making process. Moreover, if the
encapsulated lysin is added at the beginning of the cheese-making
process, e.g. while adding the cheese starter culture to the milk, about
90% of it is removed with the whey. Thus one has to add about tenfold the
required effective amount, which is economically not attractive.
10   In a later publication C.A. Shearman, K. Jury & M.J. Gasson (Feb. 1992,
ref. 2) described an autolytic *Lactococcus lactis* expressing a cloned
lactococcal bacteriophage φvML3 lysin gene. In particular they stated
that

"(e)xpression of the cloned lysin did not impair the
15   ability of *Lactococcus lactis* subsp. *lactis* and
*Lactococcus lactis* subsp. *cremoris* strains to
metabolize lactose, to clot milk and produce acid
(data not shown)".

It was suggested that during the exponential phase the lysin
20   would not, or would insufficiently be expressed. It would only be
expressed in sufficient amounts to lyse an appreciable proportion of the
cells during the stationary phase, which occurs at the end of the normal
fermentation process. The article illustrates that maintenance of
transformed lactococcal strains could be a problem. Maintenance at a
25   temperature below 30°C slightly delayed the onset of lysis but at 30°C
regrowth of lysin resistant bacteria occurred. As alternative buffering
in a sucrose medium with a sucrose percentage higher than 20% was given.
This does not seem to be suitable in a process of fermentation like
cheese making where the fermentation step occurs at 30°C or higher and
30   the presence of more than 20% sucrose is not acceptable.

Furthermore, at the end of that publication it was indicated
that expression in the stationary phase is not completely controlled. In
addition the use of osmotic buffer in a cheese maturing process is
probably not very efficient timewise when taking into consideration the
35   length of time required for a Gouda cheese immersed in a brine bath to
achieve the desired degree of salt flavour the osmotic effect of salt
concentration is not going to be very quick. The cheddar cheese making
process would probably be more suitable as the salt addition step is more
efficient, however, still requires a mixing step.

Both disclosures described the use of a lysin originating from a lactococcal bacteriophage lysin, that means an enzyme produced in nature by an undesired substance like a bacteriophage, because bacteriophage contaminations are a major problem in large scale
5    industrial dairy fermentation processes.

In a review article R. Young (1992, ref. 4) gives a survey of the state of the art on bacteriophage lysis, both mechanism and regulation. Especially in the section "Lysis in Phage Infections of Gram-Positive Hosts" on pages 468-472 it was indicated that the DNA sequence
10    found by Shearman c.s. (1989, ref. 5), which DNA sequence seems to be the same as that given in ref. 1, is probably not correct and that the deduced amino acid sequence might be quite different due to a mutation causing a phase shift in the reading frame. More particularly it is speculated that the DNA sequence of the lysin gene like the pneumococcal
15    phage associated lysin genes had no signal sequence which could account for secretion across the cytoplasmic membrane, however, this was puzzling in view of the absence of a typical N-terminal signal sequence which raises the question of how the lytic enzymes escape the cytoplasm and gain access to the cell wall.

20    In the above mentioned review of R. Young (1992, ref. 4, especially on page 469 in the paragraph bridging both columns and pages 472-473 in the section HOLIN FAMILY) it is argued that an additional protein is required for the action of bacteriophage lysins on the cell wall of infected cells. This additional protein is required for the
25    access of the murein hydrolase, which is the more scientific name for the bacteriophage lysin, to its murein substrate. In that review the term "holin" was used for this additional protein. It was described in that review that the holin makes perforations in the cell wall enabling the lysins to pass the membrane so that subsequently the lysins can hydrolase
30    the murein part of the cell wall. In this specification "holin" also means a protein or peptide required for the access of a lysin to its substrate, the murein part of the cell wall.

In the Young et al review it is stated that the realignment of the Shearman sequence and assumptions of a sequencing error obscuring a
35    start codon does present a possible basis for establishing the requirement for a holin to effect the release of this phage encoded murein hydrolase. Young et al. further state "If our analysis so far has taught us anything it is that any phage with a lysozyme gene should have a holin gene". This statement is however contradicted somewhat further on

in the same article where a lys A clone of mvl which does not appear to possess a holin encoding sequence is illustrated as exhibiting lytic activity.

Young et al further examined the putative holin family and disclose 8 different proteins unrelated in primary sequence for which genetic or physiological evidence of holin function exists. Some postulations concerning structure and function are made, however nothing definite appears to be settled regarding this issue. They indicate that as these proteins are small, hydrophobic, without enzyme function and lethal this is an array of characteristics not likely to attract legions of biochemists. This field is thus illustrated as being quite complex with little factual knowledge and a deal of speculation.

In a publication of Ward c.s. (1993; ref. 6) it is also suggested that the sequence of Shearman et al. (1989; ref. 5) is probably not correct. Comparison with a very similar phage lysin gene confirmed that a frame shift in the Shearman et al. (ref. 5) sequence is needed for aligning the two DNA sequences. Moreover, this comparison teaches that the real phage lysin is encoded by an ORF that is probably 45 bases longer than disclosed by Shearman et al. (ref. 5).

C. Platteeuw and W.M. de Vos (1992, ref. 3) described the location, characterization and expression in Escherichia coli of lytic enzyme-encoding gene, lytA, of Lactococcus lactis bacteriophage φUS3. It was described that the φvML3 lysin, which is active on a wide range of lactococcal strains, lacked homology with known lytic enzymes. The bacteriophage φUS3 was identified during studying bacteriophages specific for the cheese-making strain Lactococcus lactis SK11 (NIZO). The results showed that the deduced amino acid sequence of LytA shares similarities with that of an autolysin of Streptococcus pneumonia, suggesting that the bacteriophage φUS3 encodes an amidase rather than a lysozyme-type muramidase. The above illustrates the difficulties facing a person skilled in the art wishing to isolate DNA-sequences from different organisms. The lack of information regarding sequences and the lack of homology between known sequences makes use of probes and primers derived from known sequences quite unlikely to lead to successful isolation of a correct DNA sequence encoding a holin from different organisms.

In EP-A2-0 510 907 (AFRC, M.J. Gasson, published 28 October 1992, ref. 7) the use of bacteriophages of food-contaminating or pathogenic bacteria or the lysins thereof to kill such bacteria was described. Examples included lysins from bacteriophages of Listeria

*monocytogenes* (phage φLM4) and *Clostridium tyrobutyricum* (phage φP). Also tests for bacterial contamination can be made specific for specific bacteria by using the appropriate bacteriophage or lysin thereof and determining whether cells are lysed thereby. That European patent thus describes the use of lysins obtained from phages of food-contaminating or even pathogenic bacteria, which is not desirable for food-grade applications. Moreover, the use of such lysins is further away from the subject of this invention, which will be discussed below as it does not lie in improving flavour of food products by autolysis of lactic acid bacteria.

In another aspect the invention relates to a process for inhibiting the growth of a culture of lactic acid bacteria without lysing the cells.

The growth of lactic acid bacteria can be inhibited in several ways.

For example, in normal fermentations with lactic acid bacteria, e.g. for the production of yoghurt, when a certain low pH is obtained the high amount of lactic acid stops further fermentation. The growth changes from the log phase to the stationary phase which in effect is some sort of inhibition of the growth.

Another possibility is that the nutrients become scarce and the so-called starvation occurs, because the necessary ingredients are no longer available for growth of the bacteria. This means that no further growth occurs.

Still another possibility is the effect of pasteurization or sterilization causing cell death.

## Summary of the invention

It has now been found that holin on its own already has a bacteriostatic effect on Gramnegative bacteria like *E. coli* and Grampositive bacteria like lactic acid bacteria. Thus according to a first embodiment the invention provides a process as described in claim 1, i.e. a process for inhibiting the growth of a culture of lactic acid bacteria, which process comprises the *in situ* production in the cells of the lactic acid bacteria of a holin obtainable from bacteriophages of Gram-positive bacteria, esp. from bacteriophages of lactic acid bacteria, the gene encoding said holin being under control of a first regulatable promoter, said first regulatable promoter not normally being associated with said holin gene, said holin being capable of exerting a

6

bacteriostatic effect on the cells in which it is produced by means of a
system, whereby the cell membrane is perforated, while preferably the
natural production of autolysin is not impaired.

According to a second embodiment the invention provides a
process as described in claim 2, i.e. a process according to the first
embodiment, which additionally comprises the *in situ* production in the
cells of the lactic acid bacteria of a lysin obtainable from lactic acid
bacteria other foodgrade grampositive microorganisms or their
bacteriophages, the gene encoding said lysin being under control of a
second regulatable promoter, whereby the produced lysin effects lysis of
the cells of the grampositive or gramnegative bacteria, preferably the
lactic acid bacteria.

Preferably the second regulatable promoter is the same as the
first regulatable promoter (claim 3), and more preferably the gene
encoding the holin and the gene encoding the lysin are placed under
control of the same regulatable promoter in one operon (claim 4). It is
advantageous for food fermentations when said first or second promoter or
both can be regulated by food-grade ingredients or parameters (claim 5).
The processes according to the invention can be used in the culture of
lactic acid bacteria as such, but they can also be used in a product
containing such culture (claim 6). A specific embodiment of this latter
possibility is a process in which the lactic acid bacteria culture is
used for producing a fermented food product obtainable by the
fermentative action of the lactic acid bacteria and subsequently the
lactic acid bacteria in the fermented food product are lysed (claim 7). A
specific example of such process is one in which the fermented food
product is a cheese product (claim 8). Then an additional cheese ripening
step can be carried out, whereby some of the constituents after leaving
the lysed cells will change the composition of the cheese product (claim
9).

A third embodiment of the invention relates to a process for
combatting spoiling bacteria or pathogenic bacteria, in which a lysed
culture obtained by a process according to the second embodiment of the
invention is used as a bactericidal agent (claim 10). One way of use as a
bactericidal agent is a process for improving the shelf life of a
consumer product, in which a product obtained by a process according to
either the first or the second embodiment of the invention and containing
free holin or free lysin or both is incorporated into said consumer
product in such amount that in the resulting consumer product the growth

of spoiling bacteria or pathogenic bacteria is inhibited or that their
viability is strongly reduced (claim 11). Such consumer products comprise
edible products, cosmetic products, and products for cleaning fabrics,
hard surfaces and human skin (claim 12).

Examples of such products may be bread and bread improvers;
butter, margarine and low calorie substitutes therefor; cheeses;
dressings and mayonnaise-like products; meat products; food ingredients
containing peptides; shampoos; creams or lotions for treatment of the
human skin; soap and soap-replacement products; washing powders or
liquids; and products for cleaning food production equipment and kitchen
utensils.

A _fourth embodiment_ of the invention is a process for modifying
a mixture of peptides, which comprises (1) combining a culture of lactic
acid bacteria with a mixture of peptides obtained by proteolysis of
proteins, the cells of said culture containing both a gene encoding a
holin under control of a first regulatable promoter and a gene encoding a
lysin under control of a second regulatable promoter, which second and
first promoter can be the same and which first and second promoter are
not normally associated with the respective genes, and (2) effecting
induction of the promoter or promoters for producing both the holin and
the lysin in such amounts that the cells of the lactic acid bacteria are
lysed and the contents of the cells containing peptidases will modify the
composition of the mixture of peptides (claim 13). In order to achieve
sufficient bacterium growth the host cell must not lyse too quickly,
preferably lysis will occur at the end of the log phase or commencement
of the stationary phase.

An alternative is a process for modifying a mixture of
peptides, which comprises treating a mixture of peptides obtained by
proteolysis of proteins with a lysed culture obtained by a process
according to the second embodiment of the invention (claim 14).

The proteins to be proteolysed can be, for example, milk
proteins or vegetable proteins, or both (claim 15).

Any of the above-mentioned processes as claimed in claims 1-15,
wherein the holin is encoded by a nucleic acid sequence according to any
of claims 18-20 and/or is expressed from a recombinant vector according
to any of claims 21-24 and/or is expressed by a recombinant cell
according to any of claims 25-27 fall within the intended scope of the
invention. In addition an alternative suitable embodiment of a process
according to the invention can be directed at the inducible expression of

8

a lysin having the amino acid sequence of sequence id no 7 or being a
functional equivalent thereof.

A nucleic acid sequence encoding a holin derivable from a
grampositive bacterium such as a lactic acid bacterium, in particular a
L. lactis or a bacteriophage derivable from such a grampositive bacterium
also falls within the scope of the invention. Such a nucleic acid
sequence can for example encode the amino acid sequence of sequence id no
6 or a functional equivalent thereof such as the nucleic acid sequence of
nucleotides 103-328 of sequence id no 5. Any nucleic acid sequence
according to the invention can further be operatively linked to a first
regulatable promoter, said first regulatable promoter not normally being
associated with the holin encoding sequence.

Also comprised by the invention are recombinant vectors
comprising any of the nucleic acid sequences in any of the claimed
embodiments, said vector preferably further being foodgrade. In addition
such a recombinant vector according to the invention may suitably further
comprise a nucleic acid sequence encoding a lysin, both the holin and the
lysin being derivable from a grampositive bacterium such as a lactic acid
bacterium, in particular a L. lactis or a bacteriophage derivable from
such a grampositive bacterium. a preferred embodiment of a recombinant
vector according to the invention further comprises the natural
attachment/integration system of a bacteriophage. The natural
attachment/integration system of a bacteriophage can comprise the
bacteriophage attachment site and an integrase gene located such that
integration of the holin and optionally lysin gene will occur, said
system preferably being derived from a bacteriophage that is derivable
from a food grade host cell, preferably a lactic acid bacterium. A
suitable recombinant vector according to the invention comprises the
nucleic acid sequence encoding the holin and the nucleic acid sequence
encoding the lysin operatively linked to a foodgrade inducible promoter
that can be induced via a food grade mechanism. Such a promoter system
can for example be a thermosensitive complex inducible promoter as is
disclosed in EP94201355 and is present on plasmid pIR14. A recombinant
host cell comprising a nucleic acid sequence according to any of claims
18-20 in a setting other than in its native bacteriophage and/or a
recombinant vector according to any of claims 21-24 is claimed. Any of
the abovementioned embodiments of recombinant host cell further
comprising a nucleic acid sequence encoding a lysin, said lysin
preferably being derivable from a grampositive bacterium such as a lactic

acid bacterium, in particular a L. lactis or a bacteriophage derivable
from such a grampositive bacterium, said nucleic acid sequence encoding a
lysin preferably being in a setting other than in its native
bacteriophage or bacterium is also suitable. Preferably a recombinant
5    host cell according to the invention will be a food grade host cell,
preferably a lactic acid bacterium. Most preferably the host cell is of
the same type from which the holin and/or lysin encoding nucleic acid
sequences are derived.


10   **Brief description of the drawings**


FIGURE LEGENDS belonging to the draft publication
Fig. 1. A) Schematic outline of the PCR reactions used for the
amplification of lytP, lytR, and the combination of lytP and lytR. The
15   ORF's are indicated by hatched arrows. Sequences of the amplification
primers 1-4 (lyt1-lyt4) are given in Table 2 and as sequence id no 1-4 in
the Sequence Listing. The scale is in kilobases (kb).
          B) Schematic representation of the plasmid constructions. See
for details Materials and Methods. Abbreviations: $Em^R$, erythromycin
20   resistance marker; $Amp^R$, ampicillin resistance marker; $Cm^R$,
chloramphenicol resistance marker; $P_{spac}$ is a hybrid regulatory region,
constructed by Yansura and Henner (29), which contains the RNA polymerase
recognition sequences of an early SPO1 promoter and the lac operator;
lacI, lac repressor under the control of the Bacillus lichentformis
25   penicillinase transcriptional and translational signals, indicated as $P_{pen}$
(29); $P_1$ and $P_2$, promoters $P_1$ and $P_2$ of the bacteriophage R1-t; T,
transcription terminator; ori, origin of replication; rro, R1-t repressor
gene.
Fig. 2. Alignment of ORF 23 and the L. lactis subsp. cremoris c2 lysin
30   (c2). Identical amino acid residues are indicated with asterisks,
conserved changes by dots.
Fig. 3. Nucleotide sequence of a 1200 bp DNA fragment of R1-t carrying
lytP and lytR as represented in sequence id no 5. The deduced amino acid
sequences of lytP and lytR are indicated in sequence id no 6 and 7
35   respectively. The putative ribosomal binding sites (RBS) are underlined.
Asterisks represent stop codons. The stem-loop structure downstream of
lytR is indicated by solid arrows.
Fig. 4. Analysis of the lytic activity of the lytR gene product. Cell
free extracts of E. coli cells carrying the plasmid pAG58 (lanes 1 and 2)

or pAG58R (lanes 3 and 4) were obtained two hours after the addition of
IPTG. Abbreviations: ni, non-induced; i, induced. The arrow indicates the
position of a clearing zone as a result of lytic activity exhibited by
the *lytR* gene product.

5    Fig. 5. A) Deduced amino acid sequence of the *lytP* gene product (sequence
id no 6). Predicted transmembrane segments are indicated by bars, the
predicted ß-turn region by t's. Charged amino acid residues are indicated
+ or -, depending on the sign of the charge.

      B) Topological model of LytP based on the computer predictions.
10   The membrane-spanning amino acids are indicated.


Fig. 6. A) The effect of expression of *lytP*, *lytR*, or the combination of
*lytP* and *lytR* on the optical density of *E. coli* MC1000 cells. Optical
density measurements of *E. coli* cells carrying either pAG58 (a), pAG58R
15   (b), pAG58P (c) or pAG58PR (d), with (•) or without (○) the addition of
the inducer (IPTG) are indicated as a function of time. The time scale is
in hours before and after the time of induction (indicated by arrow).

      B) The number of colony forming units per ml of *E. coli* cells
carrying either pAG58 (empty bars), pAG58R (checkered bars), pAG58P
20   (hatched bars), or pAG58PR (filled bars) before (left diagram), and after
two hours after induction (right diagram).
Fig. 7. The effect of the induced expression of *lytP*, *lytR*, or the
combination of *lytP* and *lytR* on the optical density of *L. lactis* subsp.
*cremoris* LL302 cells. Optical density measurements of induced *L. lactis*
25   cells carrying either pIR12 (•), pIR1P (▲), pIR1R (▲), or pIR1PR (□)
respectively, are indicated as a function of growth. The optical density
(OD) measurements of *L. lactis* carrying pIR1PR, not exposed to mitomycin
C, are represented by (○). Time scale is in hours after the time of
induction with mitomycin C (1 µg/ml).

30

      The invention is illustrated by a draft publication, which is
given below.


Inducible lysis of *Lactococcus lactis* mediated by the *Lactococcus lactis*
35   subsp. *cremoris* bacteriophage R1-t lysis functions.


SUMMARY
      This work describes the involvement of two genes of the
temperate *Lactococcus lactis* subsp. *cremoris* bacteriophage R1-t, *lytR* and

*lytP*, in the lysis of its host. The gene product of *lytR* exhibits lytic
activity as it hydrolysed *Micrococcus lysodeikticus* autoclaved cell
walls. The gene product of *lytP* is required in conjunction with *lytR* to
obtain efficient lysis *in vivo* in *Escherichia coli* as was shown by
5  induction studies monitoring the optical density as a measure of cell
lysis: expression of *lytR* alone did not cause significant lysis of *E.
coli* cells whereas simultaneous expression of *lytP* and *lytR* caused lysis
of this bacterium. LytP therefore seems to have a similar function as the
S protein of the *E. coli* phage lambda, *i.e.* rendering the murein
10  substrate accessible to the lysin.
Both *lytP* and *lytR* were subcloned in an inducible expression-vector for
*L. lactis*. Induction of both genes in *L. lactis* was shown to result in
cell lysis as monitored by a decrease in optical density.


15  INTRODUCTION
        Host cell lysis by temperate bacteriophages is accomplished by
at least two fundamentally different mechanisms (30). The small single-
stranded DNA phage φX174 encodes a protein which forms a channel to
transport complete phage particles from the cytoplasm of the host to the
20  environment (4, 7, 27). However, most of the known phages encode an
enzyme with murein-degrading activity. These so-called lysins cause
breakdown of the peptidoglycan layer which is followed by lysis of the
host and the release of the phage particles.
        Lysins of bacteriophages of Gram-negative bacteria so-far
25  characterized lack a signal sequence needed for *sec*-dependent transport
across the inner membrane. A second lysis function, encoded by a gene
located immediately upstream of the lysin gene, is required for efficient
lysis. This gene encodes a so-called holin which is believed to form
holes in the cell membrane, thereby rendering the murein substrate
30  accessible to the lysin (30).
        Until recently it was believed that in Gram-positive bacteria,
phage-mediated lysis was solely accomplished through the action of a
phage-encoded lysin. Transit of the phage-encoded lysin across the
membrane was thought to occur via the general secretory route. However,
35  the observation that a signal sequence required for this type of
transport is absent in many of the identified lysins, raised the question
how the murein-degrading activity gains access to the cell wall. There is
now growing support for the idea that many of these phages require a
second function for lysis of their Gram-positive host. Recently it was

With the use of two species-specific inducible expression
systems we show that *LytR* requires an additional gene product, specified
by *lytP* upstream of *lytR*, for efficient *in vivo* lysis of *E. coli* and *L.
lactis*.

## MATERIALS AND METHODS

**Bacterial strains, phage, plasmids, and media**

The bacterial strains, phage and plasmids used in this study
are listed in Table 1.

*E. coli* was grown in TY broth (17) or on TY broth solidified with 1.5%
agar. *L. lactis* was grown in glucose M17 broth (21), or on glucose M17
agar. Erythromycin was used at 100 µg/ml and 5 µg/ml for *E. coli* and *L.
lactis*, respectively. For *E. coli*, ampicillin and chloramphenicol were
used at a concentration of 100 µg/ml and 5 µg/ml, respectively.

## DNA techniques

Plasmid DNA was isolated essentially by the method of Birnboim
and Doly (1). Restriction enzymes, Klenow enzyme, T4 DNA ligase, and T4
DNA polymerase were obtained from Boehringer GmbH (Mannheim, Germany) and
used according to the instructions of the supplier. Synthetic
oligonucleotides were synthesized using an Applied Biosystems 381A DNA
synthesizer (Applied Biosystems Inc., Foster City, Calif.). Polymerase
chain reactions were performed using Vent polymerase (New England Biolabs
Inc., Beverly, MA.). Samples were heated to 94 °C for 2 min, after which
target DNA was amplified in 25 subsequent cycles under the following
conditions: 94 °C for 1 min; 50 °C for 2 min; 73 °C for 1 min. The
primers used for amplification are listed in Table 2 and sequence id no
1-4 of the Sequence Listing. *E. coli* was used as a host for obtaining
recombinant plasmids. Transformation of *E. coli* was performed by the
method of Mandel and Higa (12). Plasmids were introduced in *L. lactis*
subsp. *cremoris* LL302, which contains a copy of the pWV01 *repA* gene on
the chromosome to ensure efficient replication, by means of
electroporation (23). DNA and protein sequences were analyzed using the
programs developed by Staden (19). Analysis of the LytP protein was
computed with the PC/Gene program (version 6.7; IntelliGenetics, Inc.,
Geneva, Switzerland) using the membrane spanning domain search program
SOAP, or the ß-turn search program BETATURN.

Table 2. Primers used for amplification of *lytP* and *lytR*

| primer | DNA sequence (5'->3') |
|--------|----------------------|
| Lyt1 | AAAACCCGGGAAGCTTGTCGACAGCAGTGATTGGTTCAACG |
| Lyt2 | TTCTAGAAGCTTGCATGCCCCTTCTTTTTTATTATTGAC |
| Lyt3 | AAAACCCGGGAAGCTTGTCGACGATAATACAGCAAGCCTAGTC |
| Lyt4 | TTTTTCTAGAAGCTTGCATGCGAAGCGGGGTTAATTTATCC |

*Hin*dIII (AAGCTT), *Sph*I (GCATGC), and *Sal*I (GTCGAC) restriction enzyme
sites are depicted boldfaced.

## IPTG and mitomycin C induction

Overnight cultures were diluted hundred-fold in fresh glucose
M17 medium (*L. lactis*) or TY medium supplemented with 0.5% glucose (*E.
coli*) and grown until the culture reached an OD600 of 0.3 at which point
isopropyl-ß-D-thiolgalactopyranoside (IPTG) or mitomycin C (Sigma
Chemical Co., St.Louis, Mo.) was added to a final concentration of 5mM or
1 µg/ml, respectively. Before the addition of IPTG, *E. coli* cells were
collected by centrifugation and resuspended in an equal volume of TY
medium without additional glucose.

## Lytic activity assay

The lytic activity assay was performed essentially as described
by Potvin *et al.* (15) with some minor adjustments as reported by Buist *et
al.* (2).

## Plasmid constructions

The *lytP* and *lytR* containing fragments of the R1-t genome were
amplified using polymerase chain reactions (PCR's). A 4.1-kb *Xba*I/*Nhe*I
fragment of the R1-t genome containing both *lytP* and *lytR* was subcloned
in the unique *Xba*I site of pUC18 resulting in the plasmid pXNB. Using
pXNB as a template, amplification with three different primer
combinations (lyt1-lyt2, lyt3-lyt4, and lyt1-lyt4; see Fig. 1A) yielded
three DNA fragments carrying either *lytP*, *lytR*, or the combined *lytP* and
*lytR*, respectively. Following digestion with *Sph*I and *Hin*dIII these three
PCR products were subcloned in *Hin*dIII and *Sph*I restricted pAG58, which

resulted in plasmids pAG58P, pAG58R, and pAG58PR, carrying lytP, lytR,
and the combined lytP and lytR, respectively, under the control of the
IPTG inducible P~spac~ promoter (Fig. 1B). For lysis studies in L. lactis
plasmids pAG58P, pAG58R and pAG58PR were first restricted with SphI and
5    SalI. Subsequently, the DNA fragments, carrying lytP, LytR and the
combination of lytP and LytR, were first subcloned in SphI/SalI-cut
pUC18. The HindII/HindIII fragments of these three constructs, designated
pUC18P, pUC18R, and pUC18PR, were cloned in the NruI and HindIII sites of
pIR12, resulting in the plasmids pIR1P, pIR1R, and pIR1PR, containing
10   lytP, lytR, and both lytP and lytR, respectively, under the
transcriptional control of the R1-t promoter-operator region (Fig. 4B).
These plasmids were transformed to L. lactis subsp. lactis strain LL302
which contains a copy of the pWV01 repA gene on the chromosome to ensure
efficient replication of pWV01-derived plasmids.

15          The construction of plasmid pIR12 was described in a co-pending
application EP-94201353.3, filed on the same date entitled: Process for
the lysis of a culture of lactic acid bacteria by means of a lysin, and
uses of the resulting lysed culture, the specification of which is
incorporated herein by reference.

20

RESULTS
Cloning and sequence analysis of the R1-t lysis functions.
          The switch from lysogenic to lytic life cycle of the temperate
L. lactis subsp. cremoris phage R1-t will ultimately result in host cell
25   lysis caused by phage-encoded lysis function(s), followed by the release
of phage particles. Inspection of the DNA sequence of R1-t revealed that
ORF 23, which specifies a protein of 270 amino acids with a calculated
molecular weight of 30,214 Da, shows significant similarity with the
lysin genes of the L. lactis bacteriophages c2 (sequence id no 8) and
30   φML3 (26, 18). The similarity between the deduced amino acid sequence of
ORF 23 with the c2 lysin is shown in Figure 2. Moreover, ORF 23 specifies
an amino acid sequence similar to the amino acid sequences of the N-
terminal portions of the amidase Hbl of the Streptococcus pneumoniae
bacteriophage HB-3 (16), and the S. pneumoniae LytA autolysin (5).
35   Therefore, ORF 23, hereafter designated lytR (Fig. 3) (sequence id no 7),
is a likely candidate for the phage-encoded lysin gene. This was not to
be predicted as is apparent from the previously cited Young reference.
          To test this supposition, lytR was cloned into the IPTG
inducible expression-vector pAG58, resulting in pAG58R (Fig. 1B). Cell-

16

free extracts of *E. coli* cells containing pAG58R were assayed for lytic
activity on an SDS-polyacrylamide gel in which *Micrococcus lysodeikticus*
autoclaved cell walls were co-polymerized. After staining of the cell
wall-containing gel with methylene blue, a clearing zone is expected at
5    positions corresponding to lytic proteins due to the breakdown of
incorporated cell walls. As shown in Figure 4, in cell free extracts of
pAG58-containing *E. coli* cells a clearing zone was absent at the position
corresponding to a protein with the expected molecular weight of the
*lytR*-encoded protein. Cell free extracts of pAG58R-containing cells,
10   however, gave rise to a clearing zone at the expected position. A weak
clearing zone was obtained with cell free extracts of uninduced cells due
to limited expression of the *lytR* gene. Cell free extracts of induced
pAG58R-containing cells showed an extended clearing zone, which became
very large in extracts obtained two hours after induction.

15          According to the rules of Von Heijne (25), LytR does not seem
to contain a signal sequence specific for secreted proteins using the
*sec*-dependent transport system. This apparent lack of a signal sequence
has also been observed in lysins of other bacteriophages of both Gram-
negative and Gram-positive bacteria. For host cell lysis to occur these
20   phages require a protein that forms holes in the cytoplasmic membrane to
render the host cell peptidoglycan layer accessible to the lysin (30).
ORF 22, which is situated upstream of *lytR*, specifies a protein of 75
amino acids with a predicted molecular weight of 7,688 Da (sequence id no
6). Although the predicted amino acid sequence shows no similarity with
25   the putative hole-forming proteins of other phages, computer analysis of
the protein product of ORF 22, designated hereafter as *lytP*, predicted
structural similarities with these proteins. Computer analysis revealed
that the protein, specified by *lytP*, has a high probability of containing
a pair of transmembrane domains, separated by a sequence with a high
30   probability of adopting a beta turn conformation (Fig. 5). In addition it
contains a charged C terminus and is highly hydrophobic. Therefore, this
protein might function as a pore-forming protein required for the release
across the cytoplasmic membrane of the R1-t encoded LytR.


35   *LytP* and *lytR* are required for lysis in *Escherichia coli*
            To determine whether the *lytP* and *lytR* gene products are
involved in host cell lysis, *lytP*, *lytR*, and the combination of *lytP* and
*lytR* were subcloned in the inducible expression- vector pAG58, resulting
in pAG58P, pAG58R and pAG58PR, respectively (Fig. 1B). Induction studies

17

were performed with *E. coli* MC1000 carrying these plasmids to examine the effects of the expression of the cloned genes on the optical density of the cells (Fig. 6A). Induction of *lytR* expression did not cause any lysis of pAG58R-containing *E. coli* cells as was determined by optical density

5    measurements. The induction of *lytP* expression, however, almost immediately halted the increase in the optical density of pAG58P-containing cells. The expression of both *lytP* and *lytR* in *E. coli* caused lysis. Lysis occurred almost immediately after the addition of IPTG to pAG58PR-containing cells, as was demonstrated by the decrease in optical

10   density which was associated with a dramatic decrease in colony forming units (CFU's) as compared to the uninduced control (Fig. 6B). No significant difference in CFU's between cells carrying pAG58 and pAG58R was observed. However, the induction of *lytP* had a significant effect on the viability of pAG58P-containing cells. The number of CFU's dropped

15   more than 200-fold within two hours.


**Expression of *lytP* and *lytR* in *Lactococcus lactis***

         In order to examine the effects of the expression of either *lytP*, *lytR* or the combined *lytP* and *lytR* on the optical density of *L.*

20   *lactis* cells, plasmids pIR1P, pIR1R, and pIR1PR were constructed (Fig. 1B). Transcription of *lytP*, *lytR*, and both *lytP* and *lytR* in these plasmids is controlled by the regulatory region of phage R1-t, which incorporates the gene specifying the repressor (*rro*) of R1-t in addition to its cognate operator region (see Fig. 1B). Expression was induced by

25   the addition of the DNA damaging substance mitomycin C. Induction studies were performed with *L. lactis* subsp. *cremoris* LL302 cells carrying the plasmids described above. Figure 7 shows that the addition of mitomycin C to *L. lactis* cells carrying pIR12 slows down the increase in optical density similar to pIR1P-containing *L. lactis* cells. The addition of

30   mitomycin C to genetically non-modified lactococci caused lysis of a small portion of the cells (results not shown). The expression of *lytR* as well as the simultaneous expression of *lytP* and *lytR* led to a decrease in optical density, as compared to pIR12-containing cells to which mitomycin C had been added, indicating cell lysis.

35

**DISCUSSION**

         We recently determined the nucleotide sequence of the temperate *L. lactis* subsp. *cremoris* bacteriophage R1-t. On the basis of the similarity of the deduced amino acid sequence with various (auto)lysins

18

we postulated that ORF 23, designated *lytR*, could specify the phage-
encoded lysin. The *lytR* gene product would consist of 270 amino acids
with an estimated molecular weight of 30,214 Da. By assaying cell-free
extracts of *E. coli* cells expressing *lytR*, it was shown that *lytR* indeed
specified a protein with lytic activity.

The similarity of LytR is mainly limited to the C-terminal
parts of the lysins of the lactococcal bacteriophages c2 and φvML3,
whereas the N-terminal part of LytR is similar to the amino acid sequence
of the N-terminal portion of the *S. pneumoniae* LytA autolysin. It has
been proposed that LytA consists of two functional modules (16), the C-
terminal domain specifying the binding site to the murein substrate and
the N-terminal domain determining the specificity of the enzyme. Since
LytA is an *N*-acetylmuramoyl-L-alanine amidase (6), it is tempting to
speculate that LytR is also an *N*-acetylmuramoyl-L-alanine amidase.

Because of the lack of an apparent signal peptide, we
hypothesized that, like many other phage-encoded lysins, LytR needs an
additional factor in order to gain access to the cell wall. ORF 22,
designated *lytP*, which is situated immediately upstream of *lytR*, can
specify a protein of 75 amino acids with the characteristics of a so-
called holin which, for other phages, was shown to render the murein
substrate accessible to lysins which lack a signal peptide (30).

This hypothesis was corroborated by the observation that the
expression of *lytP* is indeed needed for efficient lysis of *E. coli in
vivo*. In fact, induction of *lytR* expression did not result in lysis of *E.
coli*. However, *E. coli* did lyse when, in addition to *lytR*, *lytP* was also
expressed. From these results it was concluded that the transit of LytR
across the inner membrane is dependent on the *lytP* gene product. The
induction of solely *lytP* almost immediately halted the increase in
optical density and had a dramatic effect on the viability of the induced
cells. This is probably caused by the spontaneous insertion of the
protein into the lipid bilayer, inducing nonspecific lesions in the inner
membrane and thereby dissipating the membrane potential (20). Presumably
LytP forms pores in the cytoplasmic membrane, thus allowing LytR to gain
access to the cell wall.

An inducible expression system for Lactococci recently
developed in our laboratory , made it possible to examine the effects of
expression of *lytP*, *lytR*, and the combined *lytP* and *lytR* in *L. lactis*.
Expression of the combined *lytP* and *lytR* in *L. lactis* resulted in lysis
of the cells. In contrast to *E. coli*, lysis was also observed when only

19

*lytR* was expressed. Lysis of cells solely expressing *lytR* is probably
caused by the combined effect of mitomycin C and LytR: Since mitomycin C
lyses a small proportion of the cells (results not shown), LytR is
extruded in the medium, thus acting upon the cell wall from without, and
5    masking the additional requirement for LytP to effect lysis as was the
case in *E. coli*.

For bacteriophages of both Gram-negative and Gram-positive
bacteria, a system based on a murein hydrolase and a second protein
required for the access of the hydrolase to its murein substrate, seems
10   to be a general phenomenon in lysis strategies. Recently it was shown
that, in addition to the *B. subtilis* phage φ29-encoded lysin, efficient
lysis of *E. coli* also required the gene 14 product. Also several
lactococcal bacteriophages seem to encode an additional factor needed for
host cell lysis. On the basis of structural similarity, it has been
15   postulated that the bacteriophages c2 and φvML3 encode a holin (26, 30).
The deduced amino acid sequence of ORF2 of the virulent bacteriophage
φU53, isolated from *L. lactis* SK11 (14), also shares the characteristic
structural traits of a holin, making it likely that it is involved in the
translation of the phage-encoded lysin, LytA. This report, however,
20   proves for the first time that a *Lactococcus*-specified holin is required
for phage-induced lysis.


DRAFT PUBLICATION 2


25   **Development of a food-grade, thermo-inducible lysis system using the
regulatory region and lysis functions of the temperate *Lactococcus lactis*
subsp. *cremoris* bacteriophage R1-t.**


**Introduction**

30

The system is based on the food-grade removal of most of the
genomic DNA of a temperate lactococcal bacteriophage in such a way that
an inducible regulatory region of the temperate bacteriophage is directly
placed upstream of the lysis functions encoded by the prophage. As an
35   example of the general applicability of this strategy to any prophage
with a similar genetic structure, bacteriophage R1-t was taken. To obtain
the desired deletion, plasmid pBTS1 was constructed (Figure 8). In the
future pBTS2 will be constructed in which *rro* is replaced by *rro*[ts] and
therefore can be used to make this system thermo-inducible.

Experimental procedures

*Bacterial strains, phage, plasmids, and media*

5          The bacterial strains, phage, and plasmids used in this study
are listed in Table 3. *Escherichia coli* JM101 was grown in TY broth
(Rottlander and Trautner, 1970) with vigorous agitation, or on TY agar,
at 37 °C. When needed, ampicillin, isopropyl-ß-D-thiogalactopyranoside
(IPTG) and 5-bromo-4-chloro-3-indolyl ß-galactopyranoside (X-gal) (all
10        from Sigma Chemical Co., St. Louis, MO.) were used at concentrations of
100 μg/ml, 1mM and 0.002% (wt/vol), respectively. *L. lactis* subsp.
*cremoris* was grown in M17 broth (Terzaghi and Sandine, 1975), or on M17
agar, supplemented with 0.5% glucose or lactose at 30 °C. When
appropriate, erythromycin (Boehringer Mannheim, GmbH, Germany) and X-gal
15        were used at concentrations of 5 μg/ml and 0.004% (wt/vol), respectively.

*General DNA techniques and transformation*

          General DNA techniques were performed as described by Sambrook
20        *et al.* (1989). Plasmid DNA was isolated by the method of Birnboim and
Doly (1979) and by using QIAGEN Midi-Plasmid isolation columns (Qiagen
Inc.,Chatsworth, Ca.). Restriction enzymes, alkaline phosphatase and T4
DNA ligase were obtained from Boehringer Mannheim and were used according
to the instructions of the supplier. Transformation of *E. coli* was
25        performed as described by Mandel and Higa (1970). *L. lactis* LL108 was
transformed by electroporation using a Gene Pulser (Bio-Rad Laboratories,
Richmond, Calif.), as described by Holo and Nes (1989) with the
modifications suggested by Leenhouts and Venema (1993). Electroporation
of *L. lactis* R1, R131 and R1K10 was done as decribed by van der Lelie *et*
30        *al.* (1988). Oligonucleotides were synthesized using an Applied Biosystems
381A DNA synthesizer (Applied Biosystems, Inc., Foster City, Calif.).
Polymerase chain reactions (PCR's) were performed using Vent polymerase
(New England Biolabs, Inc., Beverly, MA.). After heating of the samples
to 94 °C for two minutes, target DNA was amplified in 30 subsequent
35        cycles under the following conditions: 94 °C for 1 min; 50 °C for 2 min;
73 °C for 3 min. PCR fragments were purified using the QIAEX DNA Gel
Extraction Kit (Qiagen Inc.).

*Isolation of R1-t phage particles and DNA*

An overnight culture of *L. lactis* R1 was diluted hundred-fold
in 500 ml fresh lactose M17 medium and grown until the culture reached an
5   OD600 of 0.8 at which point mitomycin C (Sigma) was added to a final
concentration of 2.5 µg/ml. Incubation at 30 °C was continued in the dark
until lysis occurred. Cells debris was removed by centrifugation for 10
min at 6000 rpm. Phage particles were precipitated by incubation with
NaCl (0.5 M) and polyethylene glycol 6000 (10 % [wt/vol]) for three hours
10   on ice and purified by a CsCl step gradient as described by Sambrook *et
al.* (1989). The bacteriophage R1-t suspension was dialysed against
several changes of 150 mM NaCl, 15 mM trisodiumcitrate. Phage DNA was
obtained by extracting the suspension twice with phenol. The DNA solution
was subsequently dialysed against 10 mM Tris-HCl/1 mM EDTA, pH 8.0.
15

*Sequencing attB-sites*

The attachment sites *attL* and *attR* of the bacteriophage R1-t
lysogen *L. lactis* R1 were determined by means of cycle sequencing using
20   the CircumVent Thermal Cycle Dideoxy DNA Sequencing Kit with Vent (*exo⁻*)
DNA Polymerase (Biolabs, New England). Primers attBL and attBR with
flanking *Xba*I and *Pst*I sites (Table 4) were used for cloning the *attB*
site of *L. lactis* MG1363. The 272-bp PCR fragment obtained with attBL and
attBR was cut with *Xba*I and *Pst*I and cloned in the *Xba*I/*Pst*I sites of
25   pUC18 and sequenced using the dideoxy-chain-termination method (Sanger *et
al.*, 1977) and the T7 sequencing kit (Pharmacia AB, Uppsala, Sweden).

*Phage titre determination*

30   Supernatant taken from *L. lactis* R131 was diluted in 1 mM MgSO₄.
The indicator strain *L. lactis* R1K10 was grown in GM17 until the OD600
was 0.7. 2 ml of culture were centrifuged and cells were resuspended in 2
ml 1 mM MgSO₄. An aliquot of 100 µl diluted phage-particles were added to
200 µl cells. After incubation at room temperature for 20 minutes 3 ml
35   Top agar (0.7% GM17 agar, 0.25% glycine, 10 mM CaCl₂) were added, mixed,
and poored on a GM17 agar-plate (1.5%) containing glycine (0.25%) and
CaCl₂ (10 mM). The plates were incubated overnight at 30 °C and the
number of plaques were determined.

*Relysogenisation of L. lactis R1K10*

After infection of *L. lactis* R1K10 with R1-t phage particles, turbid plaques will be picked and tested for their ability to give UV-
5    induction of prophage. Centrifuged cells of exponentially growing cultures will be resuspended in 1 ml 1 mM $MgSO_4$ and irradiated with a Mineralight u.v. lamp (model UVG-54, 254 nm, 3.2 Jm-2s-1: Ultra-violet Products Inc.) for 10 seconds, then 1 ml 2 times GM17 + 10 mM $CaCl_2$ will be added. The culture will be incubated at 30 °C until lysis occurs.
10

*Mitomycin C induction*

Overnight cultures of *L. lactis* were diluted hundred-fold in fresh glucose M17 medium and grown until an OD600 of 0.3 at which point
15   mitomycin C was added to a final concentration of 1 µg/ml.

*Plasmid constructions*

The plasmid pORIR1PR was constructed in *L. lactis* by subcloning
20   the 2864-bp *Eco*RI/*Sph*I-fragment of pIR1PR into pORI280 restricted with *Eco*RI and *Sph*I (Figure 8). Homology analysis showed that ORF 25 of the R1-t genome shared 98% identity with the integrase-gene of bacteriophage phi LC3 (Lillehaug and Birkeland, 1993) and was therefore called *intR*. The *intR* region was amplified with flanking *Sac*I and *Xba*I sites using PCR
25   and primers int1 and int2 (Table 4). Plasmid pUC18Int was constructed by cloning the resulting 1326-bp PCR-fragment digested with *Sac*I and *Xba*I into the *Sac*I/*Xba*I sites of pUC18. A 1047-bp *Hind*II fragment of pUC18Int, which contains the 5'-truncated *intR*, was subcloned into the alkaline phosphatase-treated *Sma*I-site of pUC18. Both the resulting plasmid
30   pUC18Intd and pUC18Int were constructed in *E.coli* JM101 (Figure 9). The 5'-truncated *intR* was cut out of pUC18Intd with *Eco*RI and *Bam*HI and subcloned in the *Eco*RI and *Bam*HI sites of pORR1PR, resulting in pBTS1 (Figure 8). The latter construction was done in *L. lactis* LL108.
In the future *rro* will be replaced by $rro^{TS}$ when this
35   temperature inducible repressor is available. This will be done by replacing the 946-bp *Nco*I-*Eco*RI fragment of pBTS1 with the comparable fragment containing $rro^{TS}$. This will result in pBTS2. Plasmid pIR14 deposited at the Centraal Bureau voor Schimmelcultures in Baarn, The

Netherlands comprises such a temperature sensitive *rro*. A detailed
description is given in European Patent Application 94201355.8.


**Results**

5

Plasmid pBTS1 was introduced in *L. lactis* LL108. As can be seen
in figure 10, pBTS1 is still able to give inducible lysis after mitomycin
C induction.

The initial idea was to introduce pBTS1 in *L. lactis* R1. As
10  pBTS1 cannot replicate in *L. lactis* (it lacks the gene for the plasmid
replication protein RepA) it will integrate into the chromosome of R1
under selective conditions. The integration will take place at either of
three homologous regions: the *intR*-region (A), the regulatory-region (B)
or the region of the lytic functions (C) (Figure 11). With appropriate
15  primer-sets the place of integration can be distinguished (Table 5 and
Figure 11). After the first recombination step in the regions A or C, a
second recombination step in the region C or A, respectively, will delete
the whole prophage and plasmid from the chromosome of strain R1, except
for the desired functions. These two recombination steps will place the
20  lytic functions directly under control of the regulatory region of R1-t,
in a one copy situation at a well defined and stable place in the
chromosome of *L. lactis*. If the integration takes place in region B
(regulatory region), the second recombination step will not result in the
substitution of *intR* and *rro* for the 5'-truncated *intR* and *rro*$^{TS}$ (future
25  work), respectively. The integrase deletion is needed to prevent *intR*
catalysed excision.

Because of the extremely low transformation efficiency of *L.
lactis* R1 (less than 1 transformant/µg pVE6007) we tried to cure the
strain of its natural plasmids. We succeeded in curing two plasmids of
30  approximately 50 kb and 2 kb, by growing *L. lactis* R1 on glucose and
incubation at 37 °C. The resulting strain *L. lactis* R131 was shown by UV-
induction to still contain R1-t prophage.

Sofar we have not succeeded in introducing pBTS1 into *L. lactis*
R131. It appears that the prophage is induced after the electroporation.
35  This, together with the presence of MgCl$_2$ and CaCl$_2$ in the recovery
medium, makes the cells lyse (Table 6).

Therefore, we are currently trying to obtain the pBTS1
integrant by two additional strategies. Firstly, pBTS1 and pVE6007 will
be introduced together in *L. lactis* R1K10. pVE6007 encodes a temperature

sensitive RepA protein enabling pBTS1 to replicate. After
relysogenisation of the resulting double transformant with phage R1-t,
pBTS1 will integrate into the chromosome when raising the temperature to
37 °C. The second way to obtain a 'food-grade' inducible lysis system is
5     to introduce pBTS1 and pVE6007 together in *L. lactis* MG1363. The *attB*-
region of this strain has been sequenced and appeared to have an homology
of 99% with the *attB*-region of *L. lactis* R1K10 (Figure 12). *L. lactis*
MG1363 will be transformed with R1-t DNA ligated at its cos-sites. After
*intR*-catalysed integration of the R1-t chromosome into the attachment
10    site of MG1363 (Figure 12), pBTS1 can integrate when raising the
temperature to 37 °C.

Figure legends

## Figure 8

5          Cloning scheme for the construction of pBTS1. *EmR*, erythromycin
resistance gene; *rro*, R1-t repressor gene; *po*, promoter(p1/p2)/operator
region from bacteriophage R1-t; *tec*, topological equivalent of lambda
*cro*; *lytP*, R1-t holin gene; *lytR*, R1-t lysin gene; T, transcription
terminator of *prtP*; ORI+, origin of replication of the lactococcal
10        plasmid pWV01; p32, promoter sequence of ORF32 of *L. lactis*; *lacZ*, ß-
galactosidase gene of *E. coli*; '*intR*, 5'-truncated R1-t integrase gene;
*amp*, ampicillin resistance gene. Only relevant restriction enzyme sites
are shown.

15   ## Figure 9

          Cloning scheme for the construction of pUC18Intd in which the
integrase gene of bacteriophage R1-t is 5'-truncated. *intR*, R1-t
integrase gene; *lacZ*, ß-galactosidase gene of *E. coli*; *amp*, ampicillin
resistance gene; '*intR*, 5'-truncated R1-t integrase gene. Only relevant
20   restriction enzyme sites are shown.

## Figure 10

          Effect of mitomycin C on OD600 of *L. lactis* LL108 containing
pORI13 (Leenhouts and Venema, 1993) (open square) and pBTS1 (filled
25   triangle). The cultures were induced with 1 µg/ml mitomycin C at time
zero (dotted line).

## Figure 11

          Schematic representation of the food-grade removal of most of
30   the genomic DNA of R1-t prophage in such a way that the inducible
regulatory region of the temperate bacteriophage is directly placed
upstream of the lysis functions encoded by the prophage. As an example,
the integration of pBTS1 in the integrase gene is depicted and the second
recombination occurs in the region of the lytic functions.
35   (A), integrase-region; (B), regulatory-region; (C), region of the lytic
functions; *attR*, 'right' phage-host junction; *intR*, R1-t integrase gene;
*rro*, R1-t repressor gene; *tec*, topological equivalent of lambda *cro*;
PROPHAGE, genomic DNA of R1-t prophage; *lytP*, R1-t holin gene; *lytR*, R1-t
lysin gene; *attL*, 'left' phage-host junction; *EmR*, erythromycin resis-

tance gene; *lacZ*, ß-galactosidase gene of *E. coli*; '*intR*, 5'-truncated
R1-t integrase gene. The genes derived from the plasmid pBTS1 are shaded.
The primers: attBR, R100, attBL and R40 are listed in table 4.


5    **Figure 12**

            Comparison of the *attB* regions of  *L. lactis* R1K10 and MG1363.
The *attB* site is shaded. Asterisks indicate identical nucleotides.


**TABLE 3. Bacterial strains, phages, and plasmid**

10   -------------------------------------------------------------------------------------

| Bacterial strain, phage or plasmid | Relevant characteristic(s) | Source or reference |
|---|---|---|
| **Bacterial strains** | | |
| *L. lactis* subsp. *cremoris* | | |
| LL108 | MG1363 (plasmid-free strain) carrying the pWV01 *repA* gene, and the *Cm$^r$* gene on the chromosome (high copy) | Leenhouts, unpublished results |
| R1 | Original R1-t lysogenic *L. lactis* subsp. *cremoris* strain | Lowrie, 1974 |
| R131 | *L. lactis* R1 cured of two natural plasmids | This study |
| R1K10 | R1-t indicator strain | lab collection |
| *Escherichia coli* JM101 | *supE thi* Δ(*lac-proAB*) [F *traD36 proAB$^+$ lacI$^q$ lacZ*ΔM15] | Messing, 1979 |
| Bacteriophage R1-t | type P335, small isometric temperate lactococcal phage, isolated from *L. lactis* subsp. *cremoris* R1 | Lowrie, 1974 |
| **Plasmids** | | |
| pIR1PR | Em$^r$ | Nauta *et al.*, 1994$^{a,b}$ |
| pORI280 | Em$^r$ | Leenhouts and Venema, 1993 |
| pORIR1PR | Em$^r$ | This study |
| pUC18 | Ap$^r$ | Yanisch-Perron *et al.*, 1985 |
| pUC18Int | Ap$^r$ | This study |
| pUC18Intd | Ap$^r$ | This study |
| pBTS1 | Em$^r$ | This study |
| pVE6007 | Cm$^r$ | Maguin *et al.*, 1992 |
| pORI13 | Em$^r$ | Leenhouts and Venema, 1993 |

TABLE 4. Nucleotide sequences of PCR primers

---

| Primer | Sequence [*] | |
|---|---|---|
| int1(SacI) | 5'-GCGCGAGCTCCCGCTCAAGTTTGACGACAAGGG-3' | (SEQ id no 9) |
| int2(XbaI) | 5'-GCGCTCTAGAGGATAGATGTGCTTTAGATAATGGC-3' | (SEQ id no 10) |
| attBL(XbaI) | 5'-GCGCTCTAGACAGCTATTCTATCTGTTCGTAAGGG-3' | (SEQ id no 11) |
| attBR(PstI) | 5'-GCGCCTGCAGTACCTAAGCACACGAAGGCCTAGG-3' | (SEQ id no 12) |
| R40 | 5'-CAAATTGGATAGTTAAGG-3' | (SEQ id no 13) |
| R100 | 5'-CTCGTGATTACTATTGG-3' | (SEQ id no 14) |

---

[*] The restriction enzyme sites are underlined.

TABLE 5. Primer-sets to check integration

---

| Strain | Primer-sets[1, 2] | |
|---|---|---|
| | attBR/R100 | attBL/R40 |

---

| | | |
|---|---|---|
| L. lactis R1 | 2271 bp | – |
| pBTS1 integrated in: | | |
| intR-region (A) | 1687 bp | – |
| regulatory-region (B) | 2271 bp | – |
| lytic-region (C) | 2271 bp | 2249 bp |
| After second recomb. | 1687 bp | 2249 bp |

---

[1] Primers are listed in Table 4.

[2] The sizes of the expected PCR-fragments are given.

TABLE 6. R1-t induction by electrical pulse

| Electrical pulse (kV)[1] | Medium[2] | 1.5 hrs | OD600 of R131[3] | | PFU/ml |
|---|---|---|---|---|---|
| | | | 4.5 hrs | 21.5 hrs | 4.5 hrs |
| 0 | GSM17 | 0.323 | 1.097 | 2.248 | $1.4 \cdot 10^4$ |
| 2.5 | GSM17 | 0.228 | 0.783 | 2.000 | $4.4 \cdot 10^5$ |
| 0 | GSM17MC | 0.242 | 0.530 | 1.120 | $1.1 \cdot 10^6$ |
| 2.5 | GSM17MC | 0.190 | 0.189 | 0.107 | $1.9 \cdot 10^7$ |

[1] Electroporation cuvette (2-mm electrode gap); 25 $\mu$F/200$\Omega$

[2] Holo and Ness, 1989.

[3] 40 $\mu$l competent cells (van der Lelie et al., 1988), cells harsvested at OD600 = 0.714

SEQUENCE LISTING


(1) GENERAL INFORMATION:

    (i) APPLICANT:
        (A) NAME: Quest International
        (B) STREET: Huizerstraatweg 28
        (C) CITY: Naarden
        (E) COUNTRY: The Netherlands
        (F) POSTAL CODE (ZIP): 1411 GP
        (G) TELEPHONE: 02159 - 99111
        (H) TELEFAX: 02159 - 46067


    (ii) TITLE OF INVENTION: Process for inhibiting the growth of a
         culture of lactic acid bacteria, and optionally lysing the
         bacterial cells, and uses of the resulting lysed culture.


    (iii) NUMBER OF SEQUENCES: 8


    (iv) COMPUTER READABLE FORM:
         (A) MEDIUM TYPE: Floppy disk
         (B) COMPUTER: IBM PC compatible
         (C) OPERATING SYSTEM: PC-DOS/MS-DOS
         (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)


    (v) CURRENT APPLICATION DATA:
        APPLICATION NUMBER:


(2) INFORMATION FOR SEQ ID NO: 1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 41 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear


    (ii) MOLECULE TYPE: DNA (genomic)

              (A) ORGANISM: Lactococcus phage R1-t

              (C) INDIVIDUAL ISOLATE: primer LYT3


          (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

5

AAAACCCGGG AAGCTTGTCG ACGATAATAC AGCAAGCCTA GTC                43



          (2) INFORMATION FOR SEQ ID NO: 4:

10

              (i) SEQUENCE CHARACTERISTICS:

                  (A) LENGTH: 41 base pairs

                  (B) TYPE: nucleic acid

                  (C) STRANDEDNESS: double

15                (D) TOPOLOGY: linear


          (ii) MOLECULE TYPE: DNA (genomic)


          (vi) ORIGINAL SOURCE:

20                (A) ORGANISM: Lactococcus phage R1-t

                  (C) INDIVIDUAL ISOLATE: primer LYT4


          (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:


25   TTTTTCTAGA AGCTTGCATG CGAAGCGGGG TTAATTTATC C           41



          (2) INFORMATION FOR SEQ ID NO: 5:


30            (i) SEQUENCE CHARACTERISTICS:

                  (A) LENGTH: 1200 base pairs

                  (B) TYPE: nucleic acid

                  (C) STRANDEDNESS: double

                  (D) TOPOLOGY: linear

35

          (ii) MOLECULE TYPE: DNA (genomic)


          (vi) ORIGINAL SOURCE:

              (A) ORGANISM: Lactococcus phage R1-t

32

(C) INDIVIDUAL ISOLATE: Fig.3 cds lytP and cds lytR

(ix) FEATURE:
    (A) NAME/KEY: CDS
5   (B) LOCATION: 103..328

(ix) FEATURE:
    (A) NAME/KEY: CDS
    (B) LOCATION: 331..1141
10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

TCTACAGGTA CATGGGAAAA TATCGGTTCA GCAGTGATTG GTTCAACGAC AATATATTAT          60

15  TGGAAACGAA CTGCATAAAA AATAAAAAAT AGGAGAAAGA AC ATG AAA ACA TTT           114
                                                   Met Lys Thr Phe
                                                    1

    TTT AAA GAT ATG GCA GAA CGT GCC ATT AAA ACA TTT GCA CAA GCA ATG         162
20  Phe Lys Asp Met Ala Glu Arg Ala Ile Lys Thr Phe Ala Gln Ala Met
     5                  10              15                  20

    ATT GGC GCT TTG GGT GCT GGT GCC ACA GGC TTA ATT GGG GTT GAT TGG         210
    Ile Gly Ala Leu Gly Ala Gly Ala Thr Gly Leu Ile Gly Val Asp Trp
25                  25              30                  35

    CTT CAA GCC TTG AGT ATC GCA GGG TTT GCA ACA GTG GTA TCA ATT CTT         258
    Leu Gln Ala Leu Ser Ile Ala Gly Phe Ala Thr Val Val Ser Ile Leu
                    40              45                  50
30

    ACT TCA TTA GCA AGT GGG ATT CCG GGC GAT AAT ACA GCA AGC CTA GTC         306
    Thr Ser Leu Ala Ser Gly Ile Pro Gly Asp Asn Thr Ala Ser Leu Val
                55              60                  65

35  AAT AAT AAA AAA GAA GGG GAA T AA ATG ACA ATT TAC GAC AAA ACG TTC        354
    Asn Asn Lys Lys Glu Gly Glu     Met Thr Ile Tyr Asp Lys Thr Phe
                70              75      1               5

33

CTA CTC GGC ACA GGT CAA GGT TCG TCA CAA AAG GCG AGT AAT CGA TAT          402
Leu Leu Gly Thr Gly Gln Gly Ser Ser Gln Lys Ala Ser Asn Arg Tyr
        10              15              20

5   ATC GTG ATT CAC GAT ACC GCC AAT GAT AAT AAC CAA GGT GAT AAT AGT      450
    Ile Val Ile His Asp Thr Ala Asn Asp Asn Asn Gln Gly Asp Asn Ser
        25              30              35              40

    GCC ACA AAT GAA GCG AGT TAT ATG CAC AAT AAC TGG CAA AAT GCC TAT      498
10  Ala Thr Asn Glu Ala Ser Tyr Met His Asn Asn Trp Gln Asn Ala Tyr
                45              50              55

    ACT CAT GCC ATT GCT GGC TGG GAT AAA GTG TAT TTG GTA GGA GAA CCT      546
    Thr His Ala Ile Ala Gly Trp Asp Lys Val Tyr Leu Val Gly Glu Pro
15              60              65              70

    GGA TAT GTT GCT TAT GGT GCA GGG AGT CCA GCT AAT GAA CGC TCA CCG      594
    Gly Tyr Val Ala Tyr Gly Ala Gly Ser Pro Ala Asn Glu Arg Ser Pro
                75              80              85
20

    TTC CAA ATC GAA CTC TCT CAC TAT TCA GAC CCA GCT AAA CAA CGT TCT      642
    Phe Gln Ile Glu Leu Ser His Tyr Ser Asp Pro Ala Lys Gln Arg Ser
        90              95              100

25  TCA TAT ATC AAC TAT ATC AAT GCT GTG CGT GAA CAA GCA AAA GTA TTC      690
    Ser Tyr Ile Asn Tyr Ile Asn Ala Val Arg Glu Gln Ala Lys Val Phe
    105             110             115             120

    GGT ATC CCT CTT ACT CTT GAT GGA GCA GGT AAT GGT ATC AAA ACT CAT      738
30  Gly Ile Pro Leu Thr Leu Asp Gly Ala Gly Asn Gly Ile Lys Thr His
                125             130             135

    AAA TGG GTT TCG GAT AAC CTT TGG GGA GAC CAT CAA GAC CCT TAC TCT      786
    Lys Trp Val Ser Asp Asn Leu Trp Gly Asp His Gln Asp Pro Tyr Ser
35              140             145             150

    TAT TTA ACA CGC ATT GGT ATT AGC AAA GAC CAA CTC GCC AAA GAC TTA      834
    Tyr Leu Thr Arg Ile Gly Ile Ser Lys Asp Gln Leu Ala Lys Asp Leu
        155             160             165

```
GCA AAC GGT ATT GGT GGG GCA TCG AAA TCT AAT CAA TCT AAT AAC GAT        882
Ala Asn Gly Ile Gly Gly Ala Ser Lys Ser Asn Gln Ser Asn Asn Asp
        170             175             180


GAT TCA ACA CAC GCA ATC AAC TAC ACA CCT AAC ATG GAG GAA AAA GAA        930
Asp Ser Thr His Ala Ile Asn Tyr Thr Pro Asn Met Glu Glu Lys Glu
    185             190             195             200


ATG ACT TAT CTT ATT TTT GCA AAA GAC ACT AAA CGC TGG TAC ATC ACA        978
Met Thr Tyr Leu Ile Phe Ala Lys Asp Thr Lys Arg Trp Tyr Ile Thr
            205             210             215


AAC GGT ATT GAA ATC CGT TAT ATC AAA ACT GGT AGA GTT CTT GGA AAT        1026
Asn Gly Ile Glu Ile Arg Tyr Ile Lys Thr Gly Arg Val Leu Gly Asn
            220             225             230


TAT CAA AAT CAA TGG TTG AAA TTC AAA CTT CCT GTG GAT ACT ATG TTC        1074
Tyr Gln Asn Gln Trp Leu Lys Phe Lys Leu Pro Val Asp Thr Met Phe
            235             240             245


CAA GCA GAA GTC GAT AAA GAG TTT GGA ACT GGA GCA ACA AAT CCA AAT        1122
Gln Ala Glu Val Asp Lys Glu Phe Gly Thr Gly Ala Thr Asn Pro Asn
        250             255             260


CGT GAC ATT TCA AAA GGA T AAATTAACCC CGCTTCGGCG GGTGTTTTTT            1171
Arg Asp Ile Ser Lys Gly
265             270


TAAATATAAT TTATTCAAAT AACATTTTT                                        1200
```

(2) INFORMATION FOR SEQ ID NO: 6:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 75 amino acids
            (B) TYPE: amino acid
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:


Met Lys Thr Phe Phe Lys Asp Met Ala Glu Arg Ala Ile Lys Thr Phe
 1            5                10               15

Ala Gln Ala Met Ile Gly Ala Leu Gly Ala Gly Ala Thr Gly Leu Ile
             20               25               30

Gly Val Asp Trp Leu Gln Ala Leu Ser Ile Ala Gly Phe Ala Thr Val
          35               40               45

Val Ser Ile Leu Thr Ser Leu Ala Ser Gly Ile Pro Gly Asp Asn Thr
          50               55               60

Ala Ser Leu Val Asn Asn Lys Lys Glu Gly Glu
    65               70               75



(2) INFORMATION FOR SEQ ID NO: 7:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 270 amino acids
            (B) TYPE: amino acid
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: protein

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:


Met Thr Ile Tyr Asp Lys Thr Phe Leu Leu Gly Thr Gly Gln Gly Ser
 1            5                10               15

Ser Gln Lys Ala Ser Asn Arg Tyr Ile Val Ile His Asp Thr Ala Asn
             20               25               30

Asp Asn Asn Gln Gly Asp Asn Ser Ala Thr Asn Glu Ala Ser Tyr Met
          35               40               45

36

His Asn Asn Trp Gln Asn Ala Tyr Thr His Ala Ile Ala Gly Trp Asp
        50                  55                  60

Lys Val Tyr Leu Val Gly Glu Pro Gly Tyr Val Ala Tyr Gly Ala Gly
5       65                  70                  75                  80

Ser Pro Ala Asn Glu Arg Ser Pro Phe Gln Ile Glu Leu Ser His Tyr
                    85                  90                  95

10    Ser Asp Pro Ala Lys Gln Arg Ser Ser Tyr Ile Asn Tyr Ile Asn Ala
                        100                 105                 110

Val Arg Glu Gln Ala Lys Val Phe Gly Ile Pro Leu Thr Leu Asp Gly
            115                 120                 125
15

Ala Gly Asn Gly Ile Lys Thr His Lys Trp Val Ser Asp Asn Leu Trp
        130                 135                 140

Gly Asp His Gln Asp Pro Tyr Ser Tyr Leu Thr Arg Ile Gly Ile Ser
20    145                 150                 155                 160

Lys Asp Gln Leu Ala Lys Asp Leu Ala Asn Gly Ile Gly Gly Ala Ser
                    165                 170                 175

25    Lys Ser Asn Gln Ser Asn Asn Asp Asp Ser Thr His Ala Ile Asn Tyr
                        180                 185                 190

Thr Pro Asn Met Glu Glu Lys Glu Met Thr Tyr Leu Ile Phe Ala Lys
            195                 200                 205
30

Asp Thr Lys Arg Trp Tyr Ile Thr Asn Gly Ile Glu Ile Arg Tyr Ile
        210                 215                 220

Lys Thr Gly Arg Val Leu Gly Asn Tyr Gln Asn Gln Trp Leu Lys Phe
35    225                 230                 235                 240

Lys Leu Pro Val Asp Thr Met Phe Gln Ala Glu Val Asp Lys Glu Phe
                    245                 250                 255

37

Gly Thr Gly Ala Thr Asn Pro Asn Arg Asp Ile Ser Lys Gly
            260                   265                   270


5      (2) INFORMATION FOR SEQ ID NO: 8:


          (i) SEQUENCE CHARACTERISTICS:
               (A) LENGTH: 241 amino acids
               (B) TYPE: amino acid
10             (C) STRANDEDNESS: single
               (D) TOPOLOGY: linear


          (ii) MOLECULE TYPE: protein


15     (vi) ORIGINAL SOURCE:
               (A) ORGANISM: Lactococcus lactis subsp. cremoris
               (C) INDIVIDUAL ISOLATE: Fig.2 c2 lysin


          (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:
20

     Leu Phe Pro Tyr Lys Lys Thr Ile Ile Ile Ile Gly Gly Gly Asn Ile
     1               5                   10                  15


     Lys Val Ser Gln Asn Gly Leu Asn Leu Ile Lys Glu Phe Glu Gly Cys
25                  20                  25                  30


     Arg Leu Thr Ala Tyr Lys Pro Val Pro Trp Glu Gln Met Tyr Thr Ile
                    35                  40                  45


30   Gly Trp Gly His Tyr Gly Val Thr Ala Gly Thr Thr Trp Thr Gln Ala
                50                  55                  60


     Gln Ala Asp Ser Gln Leu Glu Ile Asp Ile Asn Asn Lys Tyr Ala Pro
     65                  70                  75                  80
35

     Met Val Asp Ala Tyr Val Lys Gly Lys Ala Asn Gln Asn Glu Phe Asp
                    85                  90                  95

38

```
        Ala Leu Val Ser Leu Ala Tyr Asn Cys Gly Asn Val Phe Val Ala Asp
                    100                 105                 110


        Gly Trp Ala Pro Phe Ser His Ala Tyr Cys Ala Ser Met Ile Pro Lys
  5                 115                 120                 125


        Tyr Arg Asn Ala Gly Gly Gln Val Leu Gln Gly Leu Val Arg Arg Arg
                    130                 135                 140


 10     Gln Ala Glu Leu Asn Leu Phe Asn Lys Pro Val Ser Ser Asn Ser Asn
        145                 150                 155                 160


        Gln Asn Asn Gln Thr Gly Gly Met Ile Lys Met Tyr Leu Ile Ile Gly
                    165                 170                 175


 15     Leu Asp Asn Ser Gly Lys Ala Lys His Trp Tyr Val Ser Asp Gly Val
                    180                 185                 190


        Ser Val Arg His Val Arg Thr Ile Arg Met Leu Glu Asn Tyr Gln Asn
 20                 195                 200                 205


        Lys Trp Ala Lys Leu Asn Leu Pro Val Asp Thr Met Phe Ile Ala Glu
                    210                 215                 220


 25     Ile Glu Ala Glu Phe Gly Arg Lys Ile Asp Met Ala Ser Gly Glu Val
        225                 230                 235                 240


        Lys


 30
```

## List of references

1.   WO 90/00599 (AGRICULTURAL & FOOD RESEARCH COUNCIL; M.J. Gasson)
     published 25 January 1990; Uses of viral enzymes

2.   C.A. Shearman, K. Jury & M.J. Gasson (AFRC); Biotechnology 10
     (Feb. 1992) 196-199; Autolytic *Lactococcus lactis* expressing a
     lactococcal bacteriophage lysin gene

3.   C. Platteeuw and W.M. de Vos (NIZO); Gene 118 (1992) 115-120;
     Location, characterization and expression of lytic enzyme-
     encoding gene, *lytA*, of *Lactococcus lactis* bacteriophage φUS3

4.   R. Young; Microbiol. Reviews 56 (1992) 430-481; Bacteriophage
     Lysis: Mechanism and Regulation; esp. pages 468-472: Lysis in
     Phage Infections of Gram-Positive Hosts, and Perspectives

5.   C. Shearman, H. Underwood, K. Jury, and M. Gasson (AFRC); Mol.
     Gen. Genet. 218 (1989) 214-221; Cloning and DNA sequence
     analysis of a *Lactococcus* bacteriophage lysin gene

6.   L.J.H. Ward, T.P.J. Beresford, M.W. Lubbers, B.D.W.
     Jarvis and A.W. Jarvis; Can. J. Microbiol. 39 (1993)
     767-774; Sequence analysis of the lysin gene region
     of the prolate lactococcal bacteriophage c2

7.   EP-A2-0 510 907 (AGRICULTURAL & FOOD RESEARCH COUNCIL; M.J.
     Gasson) published 28 October 1992; Bacteriophage lysins and
     their applications in destroying and testing for bacteria

## Literature references of the draft publication 1

1    Birnboim, H.C., and Doly, J. (1979). A rapid alkaline
     extraction procedure for screening recombinant plasmid DNA.
     Nucleic Acids Res. 7: 1513-1523.

2    Buist, G., Haandrikman, A.J., Leenhouts, K., Venema, G., and
     Kok, J. (1994). Molecular cloning and nucleotide sequence of a
     major *Lactococcus lactis* peptidoglycan hydrolase gene (*acmA*).
     (to be published).

3    Casadaban, M.J., and Cohen, S.N.. (1980). Analysis of gene
     control signals by DNA fusion and cloning in *Escherichia coli*.
     J. Mol. Biol. 138: 179-207.

4    Denhardt, D.T., Sinsheimer, R.L. (1965). The process of
     infection with bacteriophage φX174. III. Phage maturation and
     lysis after synchronized

infection. J. Mol. Biol. 12: 641-646.

5      Diaz, E., Lopez, R., and Garcia, J.L. (1992). Role of the major pneumococcal autolysin in the atypical response of a clinical isolate of *Streptococcus pneumoniae*. J. Bacteriol. 174: 5508-5515.

6      Howard, L.V., and Gooder, H. (1974). Specificity of the autolysin of *Streptococcus (Diplococcus) pneumoniae*. J. Bacteriol. 117: 794-804.

7      Hutchison, C.A., III, and Sinsheimer, R.L. (1966). The process of infection with bacteriophage φ174. X. Mutations in a φX lysis gene. J. Mol. Biol. 18: 429-447.

8      Jaacks, K.J., Healy, J., Losick, R., and Grossman, A.D. (1989). Identification and characterization of genes controlled by the sporulation-regulatory hene *spoH* in *Bacillus subtilis*. J. Bacteriol. 171: 4121-4129.

9      Jarvis, A.W., Fitzgerald, G.F., Mata, M., et al. (1991). Species and type phages of lactococcal bacteriophages. Intervirology, 32: 2-9.

11     Lowrie, R.J. (1974). Lysogenic Strains of Group N Lactic Streptococci. Applied Microbiology, 27: 210-217.

12     Mandel, M., and Higa, A. (1970). Calcium-dependent bacteriophage DNA infection. J. Mol. Biol. 53: 159-162.

14     Platteeuw, C., and De Vos, W.M. (1992). Location, characterization and expression of lytic enzyme-encoding gene, *lytA*, of *Lactococcus lactis* bacteriophage φUS3. Gene 118: 115-120.

15     Potvin, C., Leclerc, D., Tremblay, G., Asselis, A., and Bellemare, G. (1988). Cloning, sequencing and expression of a *Bacillus* bacteriolytic enzyme in *Escherichia coli*. Mol. Gen. Genet. 214: 241-248.

16     Romero, A., Lopez, R., and Garcia, P. (1990). Sequence of the *Streptococcus pneumoniae* bacteriophage HB-3 amidase reveals high homology with the major host autolysin. J. Bacteriol. 172: 5064-5070.

17     Rottlander, E., and Trautner, T.A. (1970). Genetic and transfection studies with *Bacillus subtilis* phage SP50. J. Mol.Biol. 108:47-60.

18  Shearman, C.A., Underwood, H., Jury, K., and Gasson, M. (1989). Cloning and DNA sequence analysis of a *Lactococcus* bacteriophage lysin gene. Mol.Gen.Genet. 218: 214-221.

19  Staden, R. (1982). Automation of the computer handling of gel reading data produced by the shotgun method of DNA sequencing. Nucleic Acids Res. 10: 4731-4751.

20  Steiner, M., Lubitz, W., and Blasi, U. (1993). The missing link in phage lysis of Gram-positive bacteria: gene 14 of *Bacillus subtilis* phage φ29 encodes the functional homolog of the lambda S protein. J. Bacteriol. 175: 1038-1042.

21  Terzaghi, B.E., and Sandine, W.E. (1975). Improved medium for lactic streptococci and their bacteriophages. Appl. Microbiol. 29: 807-813.

22  Tinoco, I., Jr, Bore, P.N., Dengler, B., Levine., M.D., Uhlenbeck, O.C., Crothers, D.M., and Gralla, J. (1973). Improved estimation of secundary structure in ribonucleic acids. Nature 246: 40-41.

23  Van der Lelie, D., Van der Vossen, J.M.B.M, and Venema, G. (1988). Effect of plasmid incompatibility on DNA transfer to *Streptococcus cremoris*. Appl. Environ. Microbiol. 54: 865-871.

25  Von Heijne, G. (1986). A new method for predicting signal peptide cleavage sites. Nucleic Acids Res. 14: 4683-4690.

26  Ward, L.J.H., Beresford, T.P.J., Lubbers, M.W., Jarvis, B.D.W., and Jarvis, A.W. (1993). Sequence analysis of the lysin gene region of the prolate lactococcal bacteriophage c2. Can. J. Microbiol. 39: 767-774.

27  Witte, A., Wanner, G., Bläsi, U., Halfmann, Szostak, M., and Lubitz, W. (1990). Endogenous transmembrane tunnel formation mediated by φX174 lysis protein E. J. Bacteriol. 172: 4109-4114.

28  Yanisch-Perron, C., Vieira, J., and Messing, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33: 103-119.

29  Yansura, D.G., and Henner, D.J. (1984). Use of the *Escherichia coli lac* repressor and operator to control gene expression in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA 81: 439-443.

30  Young, R. (1992). Bacteriophage lysis: Mechanism and regulation. Microbiol. Res. 56: 430-481.

References of draft publication 2

Birnboim, H.C., and Doly, J. 1979. A rapid alkaline extraction procedure
for screening recombinant plasmid DNA. Nucleic Acids Res. 7:1513-1523.

Holo, H. and I.F. Nes. 1989. High-frequency transformation, by
electroporation, of *Lactococcus lactis* subsp. *cremoris* grown with glycine
in osmotically stabilized media. Appl. Environ. Microbiol.. 55:3119-3123.

Leenhouts, K.J. and G. Venema. 1993. Lactococcal plasmid vectors, p. 65-
94. In K. G. Hardy (ed.), Plasmids, a practical approach. Oxford
University Press, Oxford.

Leenhouts, K. J. (unpublished results)

Lillehaug, D., and N. K. Birkeland. 1993. Characterization of genetic
elements required for site-specific integration of the temperate
lactococcal bacteriophage φLC3 and construction of integration-negative
φLC3 mutants. J. of Bact.. 175:1745-1755.

Lowrie, R. J. 1974. Lysogenic strains of group N *Lactic Streptococci*.
Appl. Microbiol. 27:210-217.

Maguin, E., P. Duwat, T. Hege, D. Ehrlich, and A. Gruss. 1992. New
thermosensitive plasmid for gram-positive bacteria. Journal of Bact.
174:5633-5638.

Mandel, M., and Higa, A. 1970. Calcium-dependent bacteriophage DNA
infection. J. Mol. Biol. 53:159-162.

Messing, J. 1979. A multipurpose cloning system based on the
singlestranded DNA bacteriophage M13. Recombinant DNA technical Bulletin,
NIH Publication No. 79-99, 2, No. 2, p. 43-48.

Nauta, A., A.M. Ledeboer, G. Venema, and J. Kok. 1994[a]. Inhibiting growth
of lactic acid bacteria by a holin and optionally lysing the cells and
uses of the resulting lysed culture. European Patent aanvraag nr. EP-PA
94201354.1 (T7038)

Nauta, A., A.M. Ledeboer, G. Venema, and J. Kok. 1994[b]. Complex inducible promoter system derivable from a phage of a lactic acid bacterium and its use in a LAB for production of a desired protein. European Patent aanvraag nr. EP-PA 94201355.8 (T7039)

Rottlander, E., and T.A. Trautner. 1970. Genetic and transfection studies with *Bacillus subtilis* phage SP50. J. Mol. Biol. 108:47-60.

Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463-5467.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular cloning - a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y..

Terzaghi, B.E., and W.E. Sandine. 1975. Improved medium for lactic streptococci and their bacteriophages. Appl. Microbiol. 29:807-813.

Van der Lelie, D., J. M. B. M. van der Vossen, and G. Venema. 1988. Effect of plasmid incompatibility on DNA transfer to *Streptococcus cremoris*. Appl. Environ. Microbiol. 53:2583-2587.

Yanisch-Perron, C., J. Vieira, and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33:103-119.

C L A I M S

1.        Process for inhibiting the growth of a culture of lactic acid
bacteria,

which process comprises the *in situ* production in the cells of the lactic
acid bacteria of a holin obtainable from bacteriophages of Gram-positive
bacteria, esp. from bacteriophages of lactic acid bacteria, the gene
encoding said holin being under control of a first regulatable promoter,
said first regulatable promoter not normally being associated with the
holin gene, said holin being capable of exerting a bacteriostatic effect
on the cells in which it is produced by means of a system, whereby the
cell membrane is perforated, while preferably the natural production of
autolysin is not impaired.

2.        Process according to claim 1, which additionally comprises the
*in situ* production in the cells of the lactic acid bacteria of a lysin
obtainable from grampositive bacteria, preferably lactic acid bacteria or
their bacteriophages, the gene encoding said lysin being under control of
a second regulatable promoter, said second regulatable promoter not
normally being associated with the lysin gene whereby the produced lysin
effects lysis of the cells of the lactic acid bacteria.

3.        Process according to claim 2, in which the second regulatable
promoter is the same as the first regulatable promoter.

4.        Process according to claim 3, in which the gene encoding the
holin and the gene encoding the lysin are placed under control of the
same regulatable promoter in one operon.

5.        Process according to claim 1 or 2, in which said first or
second promoter or both are regulated by food-grade ingredients or
parameters.

6.        Process according to claim 1 or 2, in which the culture of
lactic acid bacteria is part of a product containing such culture.

7.        Process according to claim 6, in which the lactic acid bacteria
culture is used for producing a fermented food product obtainable by the
fermentative action of the lactic acid bacteria and subsequently the
lactic acid bacteria in the fermented food product are lysed.

8.        Process according to claim 7, in which the fermented food
product is a cheese product.

9.        Process according to claim 8, in which additionally a cheese
ripening step is carried out, whereby some of the constituents after
leaving the lysed cells will change the composition of the cheese product.

10.     Process for combatting spoiling bacteria or pathogenic
bacteria, in which a lysed culture obtained by a process as claimed in
claim 2 is used as a bactericidal agent.

11.     Process for improving the shelf life of a consumer product, in
which a product obtained by a process as claimed in claim 1 or 2 and
containing free holin or free lysin or both is incorporated into said
consumer product in such amount that in the resulting consumer product
the growth of spoiling bacteria or pathogenic bacteria is inhibited or
that their viability is strongly reduced.

12.     Process according to claim 11, in which the consumer product
is selected from the group consisting of edible products, cosmetic
products, and products for cleaning fabrics, hard surfaces and human
skin.

13.     Process for modifying a mixture of peptides, which comprises
(1) combining a culture of lactic acid bacteria with a mixture of
peptides obtained by proteolysis of proteins, the cells of said culture
containing both a gene encoding a holin under control of a first
regulatable promoter and a gene encoding a lysin under control of a
second regulatable promoter, which second and first promoter can be the
same, and which second and first promoters are not normally associated
with the respective genes and
(2) effecting induction of the promoter or promoters for producing both
the holin and the lysin in such amounts that the cells of the lactic acid
bacteria are lysed and the contents of the cells containing peptidases
will modify the composition of the mixture of peptides.

14.     Process for modifying a mixture of peptides, which comprises
treating a mixture of peptides obtained by proteolysis of proteins with a
lysed culture obtained by a process according to claim 2.

15.     Process according to claim 13 or 14, in which the proteins
comprise milk proteins or vegetable proteins, or both.

16.     Process according to any of the preceding claims, wherein the
holin is encoded by a nucleic acid sequence according to any of claims
18-20 and/or is expressed from a recombinant vector according to any of
claims 21-24 and/or is expressed by a recombinant cell according to any
of claims 25-27.

17.     Process according to any of claims 2-16, wherein the lysin has
the amino acid sequence of sequence id no 7 or is a functional equivalent
thereof.

18.     A nucleic acid sequence encoding a holin derivable from a grampositive bacterium such as a lactic acid bacterium, in particular a L. lactis or a bacteriophage derivable from such a grampositive bacterium.

19.     A nucleic acid sequence according to claim 18 encoding the amino acid sequence of sequence id no 6 or or a functional equivalent thereof such as the nucleic acid sequence of nucleotides 103-328 of sequence id no 5.

20.     A nucleic acid sequence according to claim 18 or 19, further being operatively linked to a first regulatable promoter, said first regulatable promoter not normally being associated with the holin encoding sequence.

21      A recombinant vector comprising a nucleic acid sequence according to any of claims 18-20, said vector preferably further being foodgrade.

22.     A recombinant vector according to claim 21 further comprising a nucleic acid sequence encoding a lysin, both the holin and the lysin being derivable from a grampositive bacterium such as a lactic acid bacterium, in particular a L. lactis or a bacteriophage derivable from such a grampositive bacterium.

23.     A recombinant vector according to claim 21 or 22 further comprising a the natural attachment/integration system of a bacteriophage for example said system comprising the bacteriophage attachment site and an integrase gene located such that integration of the holin and optionally lysin gene will occur, said system preferably being derived from a bacteriophage that is derivable from a food grade host cell, preferably a lactic acid bacterium.

24.     A recombinant vector according to any of the claims 21-23, wherein the nucleic acid sequence encoding the holin and the nucleic acid sequence encoding the lysin are operatively linked to a foodgrade inducible promoter that can be induced via a food grade mechanism, for example by being a thermosensitive complex inducible promoter.

25.     A recombinant host cell comprising a nucleic acid sequence according to any of claims 18-20 in a setting other than in its native bacteriophage and/or a recombinant vector according to any of claims 21-24.

26.     A recombinant host cell according to claim 25 further comprising a nucleic acid sequence encoding a lysin, said lysin preferably being derivable from a grampositive bacterium such as a lactic

acid bacterium, in particular a L. lactis or a bacteriophage derivable from such a grampositive bacterium, said nucleic acid sequence encoding a lysin preferably being in a setting other than in its native bacteriophage or bacterium.

27.      A recombinant host cell according to claim 25 or 26 being a food grade host cell, preferably a lactic acid bacterium, most preferably the host cell is of the same type from which the holin and/or lysin encoding nucleic acid sequences are derived
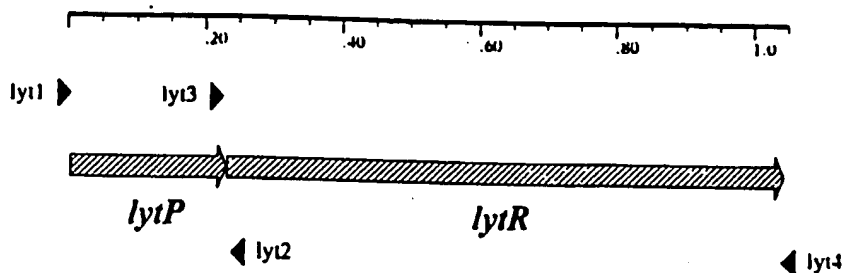
* * * * *

## Fig. 1A



## Fig. 2

```
ORF 23   MTIYDKTFLLGTGQGSSQKASNRYIVIHDTANDNNQGDNSATNEASYMHN   50
c2       LFPYKKTIII-IGGGNIKVSQNGLNLIKEFE---------GCRLTAYKPV   40
         .  *  **  ..   *  *     .   *    .*..         ..*


ORF 23   NWQNAYTHAIAGWDKVYLVGEPGYVAYGAGSPANERSPFQIELSHYSDPA   100
c2       PWEQMYT---IGWGHYGVTAGTTW------TQAQADSQLEIDINNKYAPM   81
         *  .  **    **    .  .      .     * . *  *..      *


ORF 23   KQRSSYINYINAVREQAKVFGIPLTLD-GAGNGIKTHKWVS-DNLWGDHQ   148
c2       VD-----AYVKG-KANQNEFDALVSLAYNCGNVFVADGWAPFSHAYCASM   125
              *.    .  .    *    ..*    **    .  *


ORF 23   DPYSYLTRIGISKDQLAKDLANGIGGASKSNQSNNDDSTHAINYTPNMEE   198
c2       IP-KYRNAGGQVLQGLVRRRQAELNLFNKPVSSNSNQNNQT-------GG   167
            *   *     *     *        .  *   **


ORF 23   KEMTYLIFAKD----TKRWYITNGIEIRYIKTGRVLGNYQNQWLKFKLPV   244
c2       MIKMYLIIGLDNSGKAKHWYVSDGVSVRHVRTIRMLENYQNKWAKLNLPV   217
          ***   *       * .**.. *   *  . * * .* **** * *  ***


ORF 23   DTMFQAEVDKEFGTGATNPNRDISKG   270
c2       DTMFIAEIEAEFGRKIDMASGEVK--   241
         **** **.. ***          ..
```

# Fig. 1B
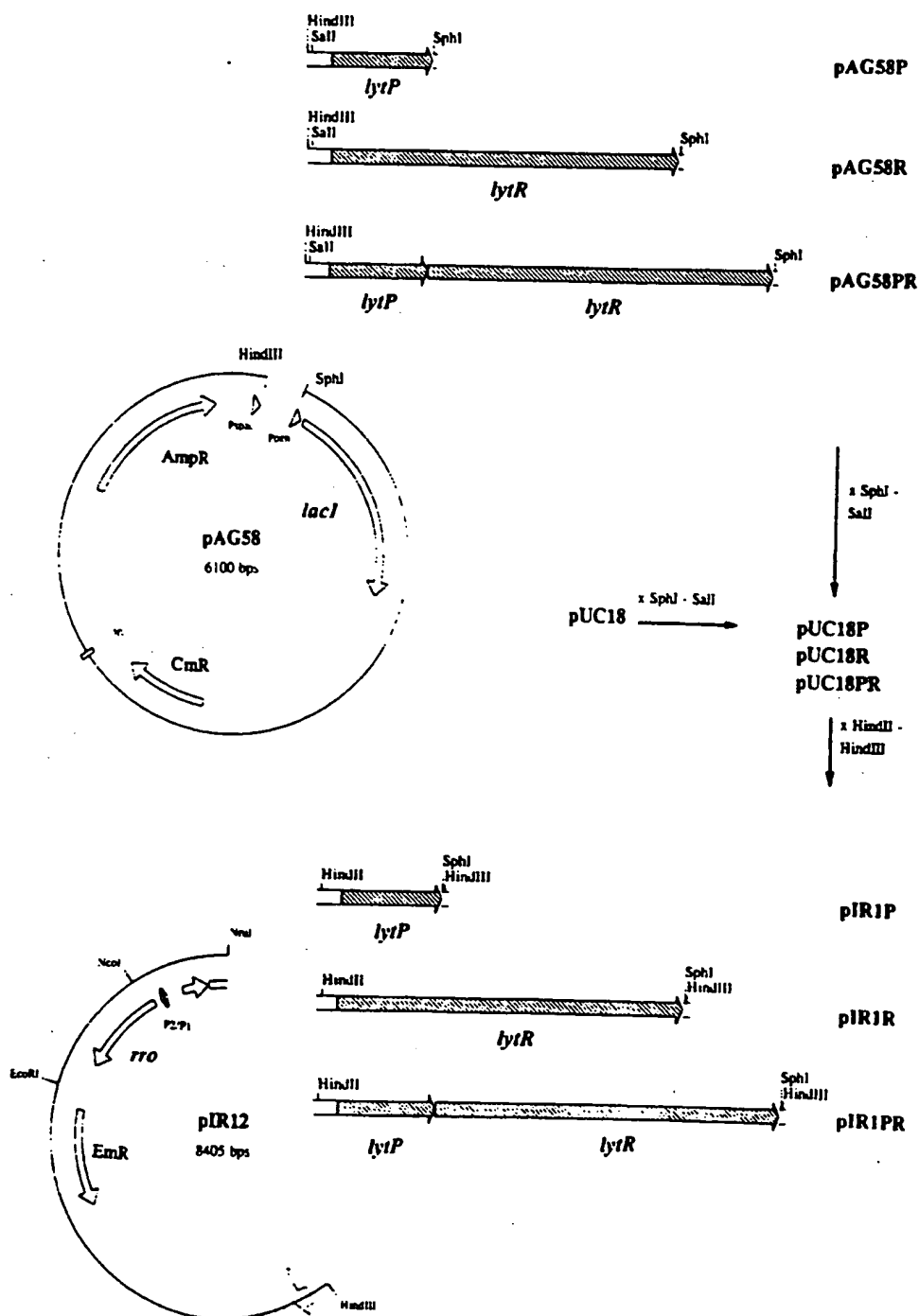
# Fig. 3

```
TCTACAGGTACATGGGAAAATATCGGTTCAGCAGTGATTGGTTCAACGACAATATATTAT

                            RBS        ---> lytP
TGGAAACGAACTGCATAAAAAATAAAAAATAGGAGAAAGAACATGAAAACATTTTTTAAA
                                           M  K  T  F  F  K

GATATGGCAGAACGTGCCATTAAAACATTTGCACAAGCAATGATTGGCGCTTTGGGTGCT
 D  M  A  E  R  A  I  K  T  F  A  Q  A  M  I  G  A  L  G  A

GGTGCCACAGGCTTAATTGGGGTTGATTGGCTTCAAGCCTTGAGTATCGCAGGGTTTGCA
 G  A  T  G  L  I  G  V  D  W  L  Q  A  L  S  I  A  G  F  A

ACAGTGGTATCAATTCTTACTTCATTAGCAAGTGGGATTCCGGGCGATAATACAGCAAGC
 T  V  V  S  I  L  T  S  L  S  S  G  I  P  G  D  N  T  A  S
                          RBS    ---> lytR
CTAGTCAATAATAAAAAAGAAGGGGAATAAATGACAATTTACGACAAAACGTTCCTACTC
 L  V  N  N  K  K  E  G  E  *  M  T  I  Y  D  K  T  F  L  L

GGCACAGGTCAAGGTTCGTCACAAAAGGCGAGTAATCGATATATCGTGATTCACGATACC
 G  T  G  Q  G  S  S  Q  K  A  S  N  R  Y  I  V  I  H  D  T

GCCAATGATAATAACCAAGGTGATAATAGTGCCACAAATGAAGCGAGTTATATGCACAAT
 A  N  D  N  N  Q  G  D  N  S  A  T  N  E  A  S  Y  M  H  N

AACTGGCAAAATGCCTATACTCATGCCATTGCTGGCTGGGATAAAGTGTATTTGGTAGGA
 N  W  Q  N  A  Y  T  H  A  I  A  G  W  D  K  V  Y  L  V  G

GAACCTGGATATGTTGCTTATGGTGCAGGGAGTCCAGCTAATGAACGCTCACCGTTCCAA
 E  P  G  Y  V  A  Y  G  A  G  S  P  A  N  E  R  S  P  F  Q

ATCGAACTCTCTCACTATTCAGACCCAGCTAAACAACGTTCTTCATATATCAACTATATC
 I  E  L  S  H  Y  S  D  P  A  K  Q  R  S  S  Y  I  N  Y  I

AATGCTGTGCGTGAACAAGCAAAAGTATTCGGTATCCCTCTTACTCTTGATGGAGCAGGT
 N  A  V  R  E  Q  A  K  V  F  G  I  P  L  T  L  D  G  A  G

AATGGTATCAAAACTCATAAATGGGTTTCGGATAACCTTTGGGGAGACCATCAAGACCCT
 N  G  I  K  T  H  K  W  V  S  D  N  L  W  G  D  H  Q  D  P

TACTCTTATTTAACACGCATTGGTATTAGCAAAGACCAACTCGCCAAAGACTTAGCAAAC
 Y  S  Y  L  T  R  I  G  I  S  K  D  Q  L  A  K  D  L  A  N

GGTATTGGTGGGGCATCGAAATCTAATCAATCTAATAACGATGATTCAACACACGCAATC
 G  I  G  G  A  S  K  S  N  Q  S  N  N  D  D  S  T  H  A  I

AACTACACACCTAACATGGAGGAAAAAGAAATGACTTATCTTATTTTTTGCAAAAGACACT
 N  Y  T  P  N  M  E  E  K  E  M  T  Y  L  I  F  A  K  D  T

AAACGCTGGTACATCACAAACGGTATTGAAATCCGTTATATCAAAAACTGGTAGAGTTCTT
 K  R  W  Y  I  T  N  G  I  E  I  R  Y  I  K  T  G  R  V  L

GGAAATTATCAAAATCAATGGTTGAAATTCAAACTTCCTGTGGATACTATGTTCCAAGCA
 G  N  Y  Q  N  Q  W  L  K  F  K  L  P  V  D  T  M  F  Q  A

GAAGTCGATAAAGAGTTTGGAACTGGAGCAACAAATCCAAATCGTGACATTTCAAAAGGA
 E  V  D  K  E  F  G  T  G  A  T  N  P  N  R  D  I  S  K  G

TAAATTAACCCCGCTTCGGCGGGGTGTTTTTTTTAAATATAATTTATTCAAATAACATTTTT
 *      ------->     <-------
```
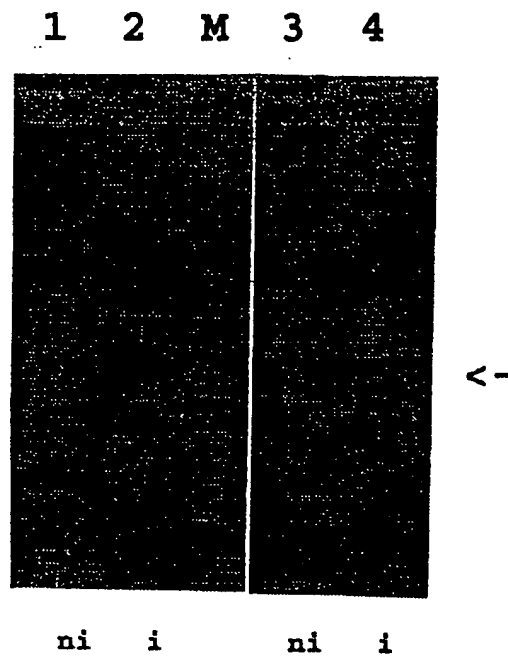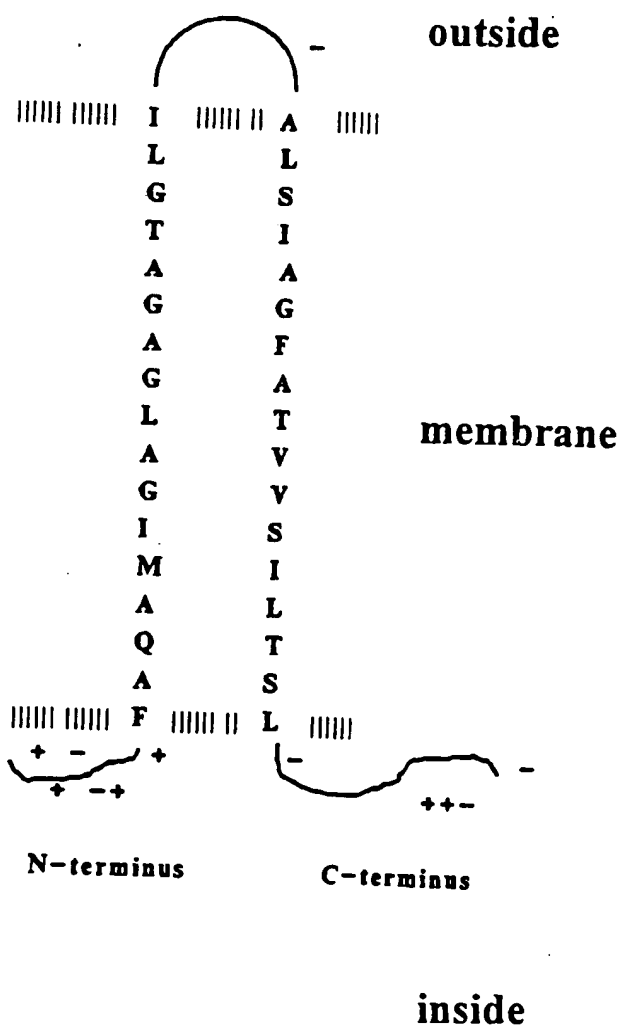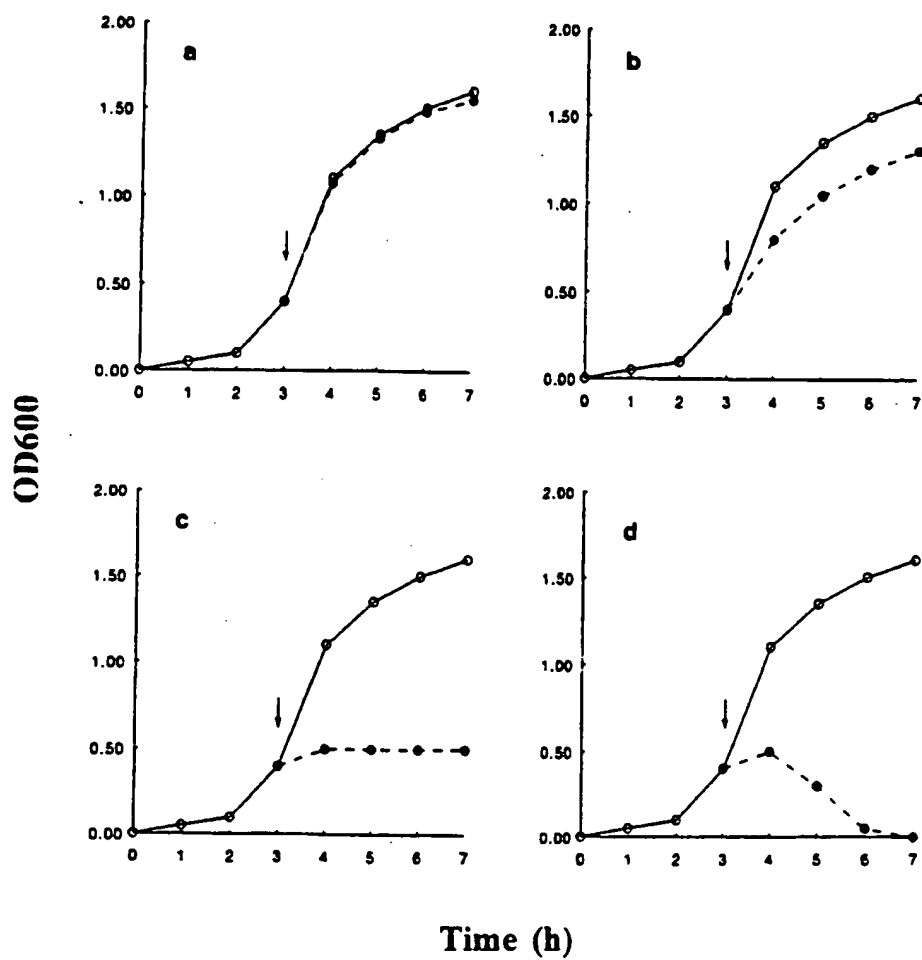
Figure 4

# Fig.5

**A**

```
        +    +-   -+  +
MKTFFKDMAERAIKTFAQAMIGALGAGATGLIGVDWLQALSIAGFATVVSILTSLASGIPGD
                                tttt

        ++- -
NTASLVNNKKEGE
```

**B**

Fig. 6A



Time (h)

## Fig. 8

Fig. 9

## Fig.10
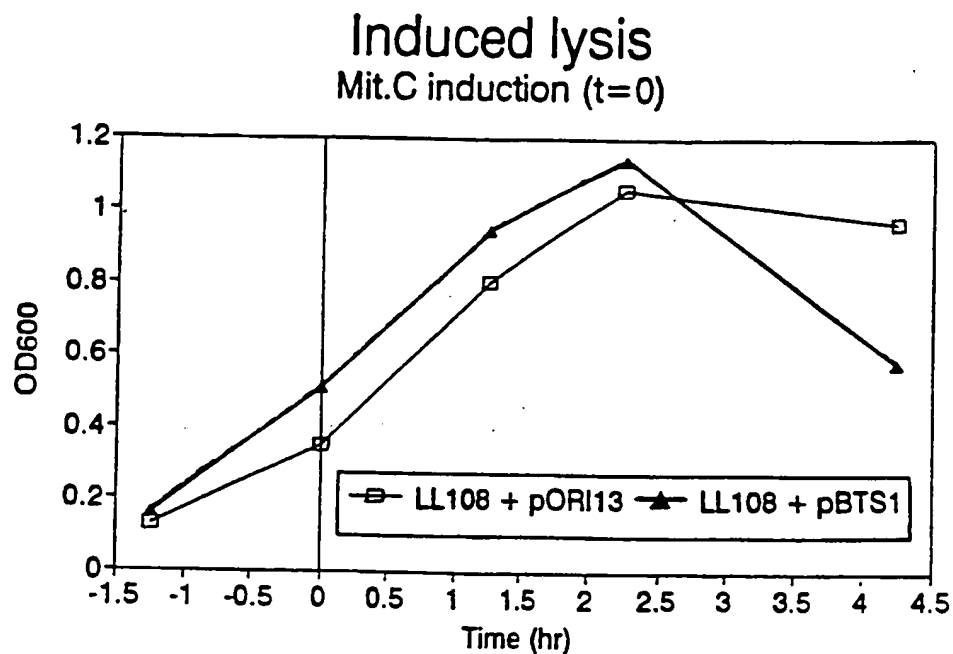
### Induced lysis
#### Mit.C induction (t=0)
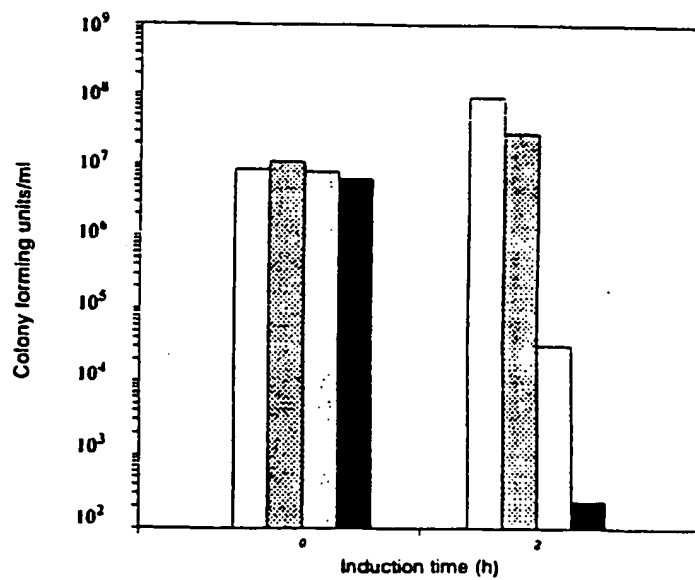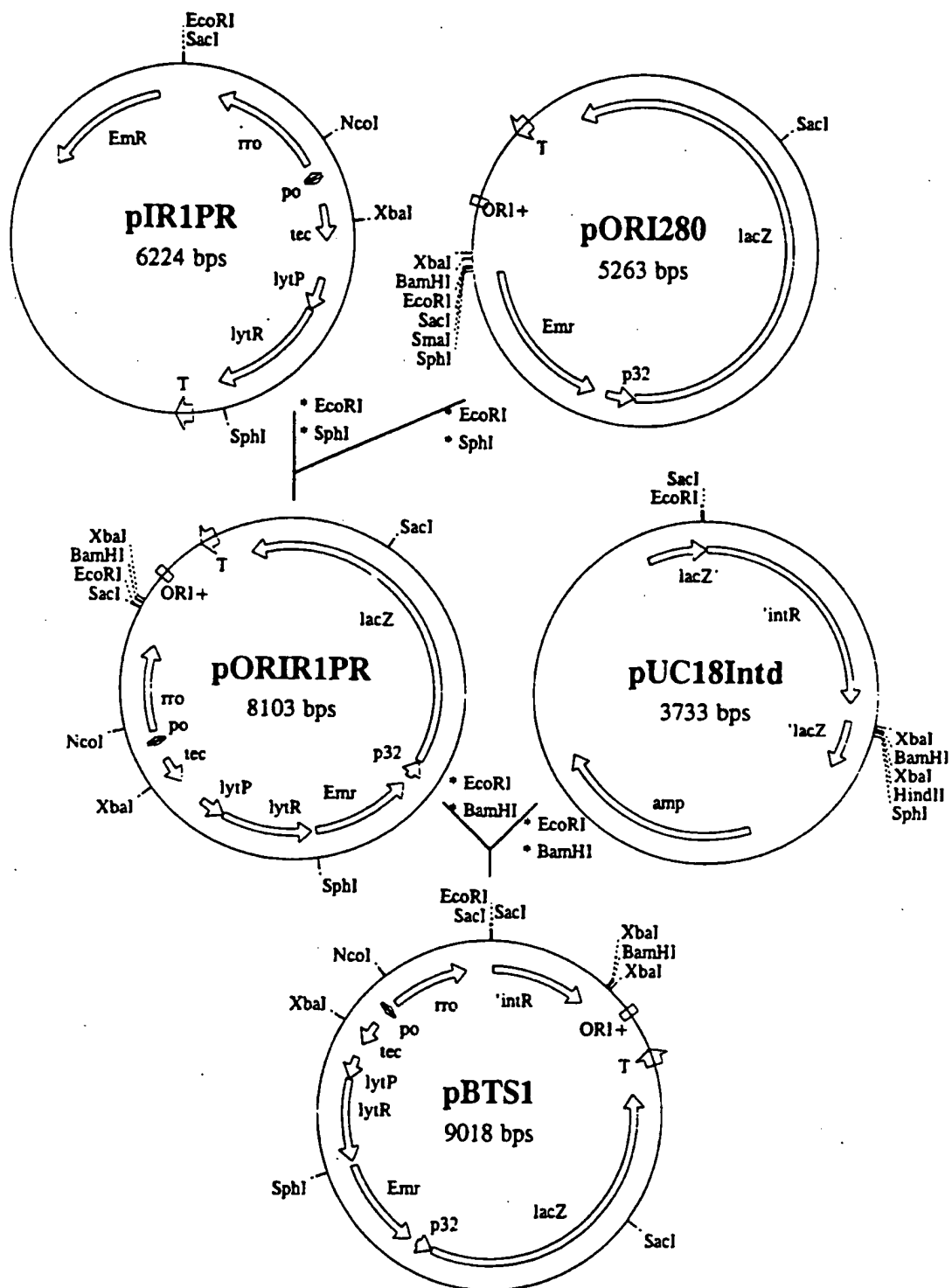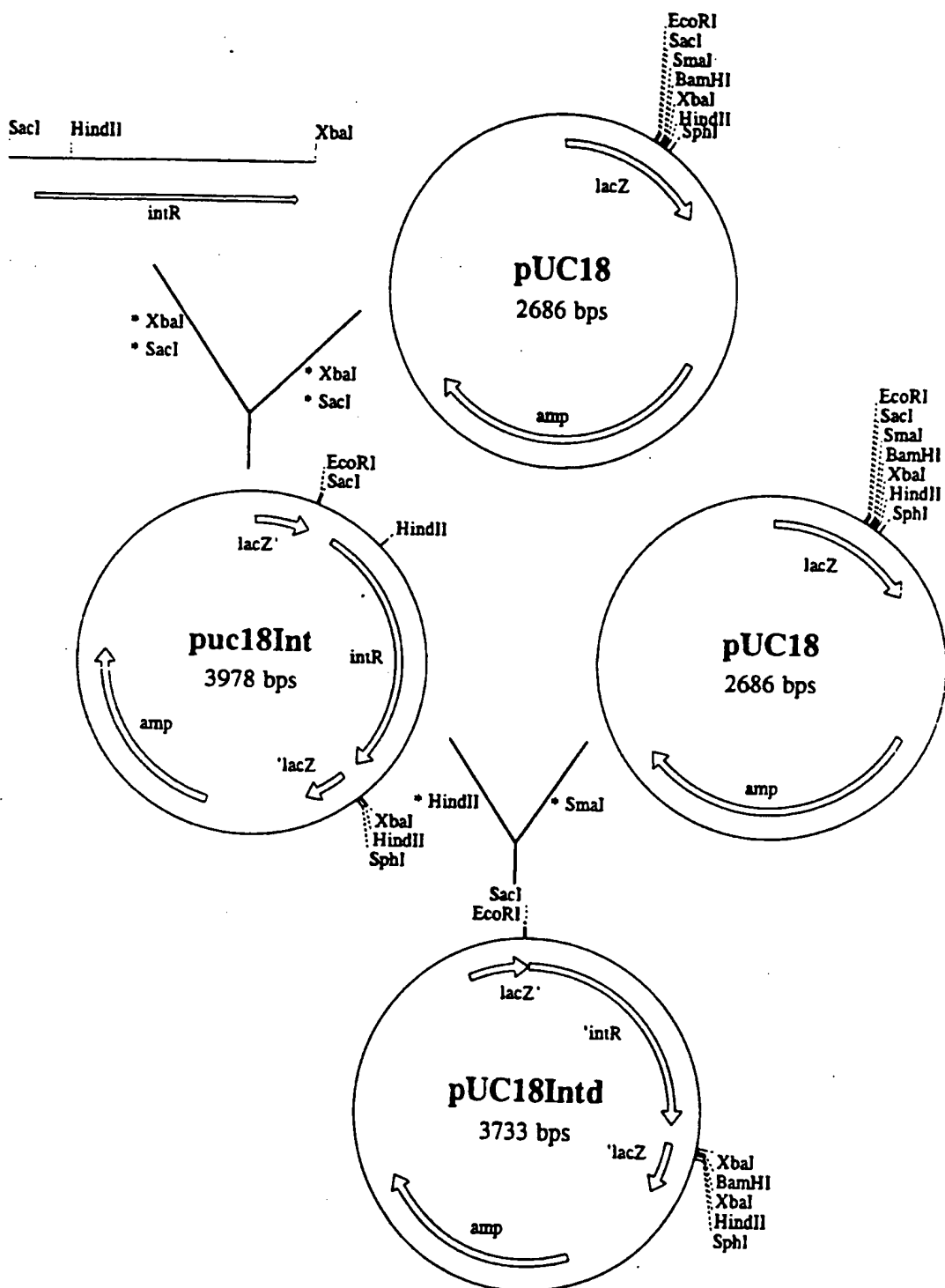


## Fig.12

```
R1K10    ACAGCTATTCTATCTGTTCGTAAGGAAAACGCTCAGGAAGTGGTTGATAC 50
MG1363   ACAGCTATTCTATCTGTTCGTAAGGAAAACGCTCAGGAAGTGGTTGATAC 50
         **************************************************


R1K10    CCTTGTTAAAGCAGGAATCAAAGGCTTTCTTAACTTTGCACCTATTCGCT 100
MG1363   CCTTGTTAAAGCAGGAATCAAAGGCTTTCTTAACTTTGCACCTATTCGCT 100
         **************************************************


R1K10    TGAAAGTCCCTTCAGATGTTGTTGTTCAATCTATTGATTTAACTAAAGAA 150
MG1363   TGAAAGTCCCTTCAGATGTTGTTGTCCAATCTATTGATTTAACTAAAGAA 150
         ************************* ************************


R1KI0    TTGCAAACCTTGTTATTCTTCATGGGAGCTCAAGAAGAATAAAAGACAAG 200
MG1363   TTGCAAACTTTGTTATTCTTCATGGGAGCTCAAGAAGAATAAAAGACAAG 200
         ********  ****************************************


R1KI0    CAAAATTTTATCAAAACTATTAATGATACCTAGGCCTTCGTGTGCTTAGG 250
MG1363   CAAAATTTTATCAAAACTATTAATGATACCTAGGCCTTCGTGTGCTTAGG 250
         **************************************************


R1K10    TA 252
MGI363   TA 252
         **
```
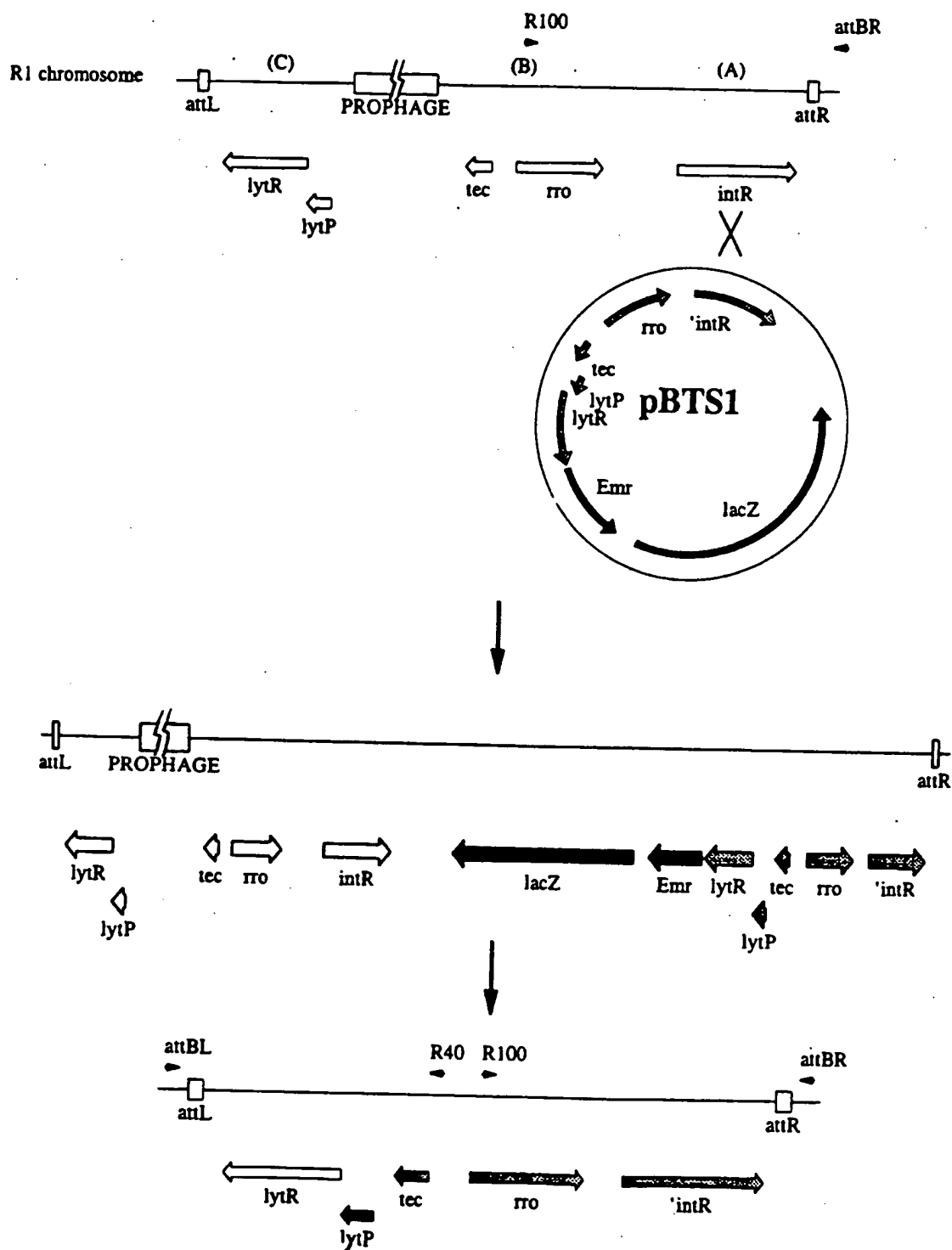
# Fig.11

# INTERNATIONAL SEARCH REPORT

**A. CLASSIFICATION OF SUBJECT MATTER**
IPC 6    C12N15/74    C07K14/315    C12N9/14    A23C19/032

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched  (classification system followed by classification symbols)
IPC 6    C12N    A23C    C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | JOURNAL OF BACTERIOLOGY, vol. 175,no. 4, 1993 pages 1038-1042, M.STEINER ET AL. 'The missing link in phage lysis of gram-positive bacteria : gene 14 of Bacillus subtilis phage sigma 29 encodes the functional homolog of lambda S protein' cited in the application | 18,21 |
| Y | see the whole document<br><br>--- <br><br>-/-- | 1-17, 21-27 |

☒ Further documents are listed in the continuation of box C.  ☒ Patent family members are listed in annex.

* Special categories of cited documents :

'A' document defining the general state of the art which is not considered to be of particular relevance

'E' earlier document but published on or after the international filing date

'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

'O' document referring to an oral disclosure, use, exhibition or other means

'P' document published prior to the international filing date but later than the priority date claimed

'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

'&' document member of the same patent family

Date of the actual completion of the international search

27 September 1995

Date of mailing of the international search report

17. 10. 95

Name and mailing address of the ISA
European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Gurdjian, D

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | JOURNAL OF GENERAL MICROBIOLOGY, vol. 138, COLCHESTER GB, page 1949 LAZAREVIC, VLADIMIR ET AL 'Sequencing and analysis of the Bacillus subtilis lytRABC divergon: a regulatory unit encompassing the structural genes of the N-acetylmuramoyl-L-alanine amidase and its modifier' see the whole document --- | 1-17, 21-27 |
| Y | BIOTECHNOLOGY, vol. 10, 1992 NEW YORK US, page 196 SHEARMAN C.A. ET AL. 'Autolytic lactococcus lactis ...' cited in the application see the whole document --- | 1-17, 21-27 |
| A | WO,A,90 00599 (AGRICULTURAL & FOOD RESEARCH COUNCIL) 25 January 1990 cited in the application see abstract; claims 1-17 --- | 7-11 |
| Y | EP,A,0 510 907 (AGRICULTURAL AND FOOD RESEARCH COUNCIL) 28 October 1992 cited in the application see claims 1-14 --- | 10-12, 21-25 |
| A | GENE., vol. 118, AMSTERDAM NL, page 115 PLATTEEUW C. ET AL. cited in the application see the whole document --- | 1-27 |
| P,X | MICROBIOLOGY, vol. 140, 1994 pages 3061-3069, C.SCHOULER ET AL. 'Sequence and organization of the Lactococcal prolate-headed bIL67 phage genome' see the whole document --- | 18,21 |
| P,X | CANADIAN J.MICROBIOL., vol. 40,no. 8, 1994 pages 658-665, N.K.BIRKELAND 'Cloning, molecular characterization, and expression of the genes encoding the lytic functions of lactococcal bacteriophage sigmaLC3 : a dual lysis system of modular design' see the whole document --- | 18,21 |

-/--

1

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| P,X | APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 60,no. 6, June 1994 pages 18785-1883, E.K.ARENDT ET AL. 'Molecular characterization of lactococcal bacteriophage Tuc 2009 and identification and analysis of genes encoding lysin, a putative holin, and two structural proteins' see the whole document ----- | 18,21 |

# INTERNATIONAL SEARCH REPORT

Intern ...al Application No
PCT/NL 95/00171

| Patent document cited in search report | Publication date | Patent family member(s) | | Publication date |
|---|---|---|---|---|
| WO-A-9000599 | 25-01-90 | AU-B- | 623166 | 07-05-92 |
| | | AU-B- | 4037789 | 05-02-90 |
| | | EP-A- | 0425572 | 08-05-91 |
| | | GB-A- | 2243611 | 06-11-91 |
| | | US-A- | 5360617 | 01-11-94 |
| EP-A-0510907 | 28-10-92 | GB-A- | 2255561 | 11-11-92 |
| | | AU-B- | 650737 | 30-06-94 |
| | | AU-B- | 1502392 | 22-10-92 |